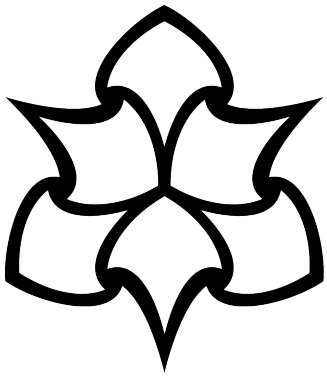

The Involvement of microRNAs in the pathogenesis of Systemic Lupus Erythematosus-related Vascular Calcification

MSc (by Research)

2018

School of Healthcare Science

Shikha M Tandel

A thesis submitted in fulfilment of the requirements of the Manchester Metropolitan University for the degree of Master of Science (by Research)

Contents

Table of Figures

Table of Tables

Abstract

Background Patients with Systemic Lupus Erythematosus (SLE), a systemic autoimmune disease, have an elevated risk of developing cardiovascular disease compared to their healthy counterparts. The chronic inflammatory state in these patients heighten their risk of developing premature atherosclerosis and subsequent progression to coronary artery calcification (CAC). CAC increases the risk of coronary events occurring and it is characterised by the osteogenic transdifferentiation of coronary artery smooth muscle cells (CASMCs) and consequent deposition of hydroxyapatite minerals within the extracellular matrix. Runx2 is a potent regulator of osteogenic differentiation of CASMCs and its expression is regulated by microRNAs (miRNA or miR). CAC is a major clinical problem, but limited treatment options are available. This study investigates whether miRNA alteration affects mineralisation in an *in vitro* calcification assay model.

Aims This study aimed to validate the association of miR-3148 with Runx2-driven CAC and investigate the effects of different miRNAs on genes involved in CAC in CASMCs.

Methods Human Embryonic Kidney (HEK) 293T cells were used to produce high-titre lentiviral vectors for miR-3148 and its sponge, and pLL3.7 plasmid. Human Coronary Artery Smooth Muscle Cells (CASMCs) were transfected with lentivirus and cultured in osteogenic media. The level of miRNA-3148 in cells and microvesicles derived from TNF-α stimulated CASMCs *in vitro* was determined. Alizarin Red S staining and quantification was used to identify changes in mineralisation between the different groups. Gene expression analysis was conducted.

Results miR-3148 levels were higher in TNF-α stimulated CASMCs compared to control. Stable transfection was observed in lentivirus transfected cells. A lower level of calcium deposits was observed in cells transfected with miR-3148 lentivirus in comparison to empty vector control (*P = 0.00201*) and transfection with miRNA-3148 sponge. miRNA-3148 also lowered Runx2-gene expression in relation to control and miR-3148 sponge at day 4.

Conclusion The current study demonstrates that overexpression of miR-3148 reduces the level of mineralisation via Runx2 *in vitro*, suggesting a protective role for miRNA-3148 against osteogenic transdifferentiation of CASMCs. These data imply the possibility of using microvesicles and/or miRNAs for drug therapy in the management or treatment of CAC.

Acknowledgments

I would like to thank my supervisors, Prof Yvonne Alexander and Dr Fiona Wilkinson, for the support and guidance with their expert knowledge in the field and for the encouragement throughout my time as their student. This project was supported by the Cardiovascular Research group in the Healthcare Science School of Manchester Metropolitan University. I feel lucky to have had the chance to work in this group which also gave me an opportunity to work among experienced lab users who were very helpful in answering my questions and queries.

With special thanks to Dr Alex Langford-Smith for his patient guidance, helping me in designing the experiments and training me in using the instruments and equipment needed to conduct my experiments.

Further thanks to the postgraduate students and technicians who helped to solve my queries and difficulties in the laboratory and while conducting experiments. In particular Francesca Bartolli-Leonard, Nicola Edwards, Cai Astley, Stuart Fielding, Glen Ferris and Dr Gary Sidgewick. I appreciate the help and resources provided by Professor Tristan McKay's group.

Finally, I would like to express my gratitude towards my family and friends for supporting me and always motivating me to do my best.

1 Introduction

1.1 Systemic Lupus Erythematosus and Cardiovascular Disease

Systemic Lupus Erythematosus (SLE) is a prototypic, systemic autoimmune disease that mainly affects women of childbearing age, with significant mortality and morbidity. An elevation in the prevalence of cardiovascular disease (CVD) has been observed in patients with SLE, and it is the leading cause of death in this patient group (Sinicato et al., 2013). A recent study shows a 2.7-fold increased risk of an SLE patient developing acute CVD events relative to the expected value based on a Framingham risk score (Magder and Petri, 2012). The reason for this heightened rate of CVD is not completely understood, but factors contributing to CVD include medications such as glucocorticoids, inflammation and the effects of cytokines (Sinicato et al., 2013). Results from our group have highlighted significant factors that should be taken into consideration when treating SLE patients (Edwards et al., 2018).

CVDs refer to dysfunctions in the heart or vasculature, including coronary heart disease, hypertension, congenital heart disease, peripheral artery disease and heart failure. Dyslipidaemia (observed in 36% to over 60% of SLE patients) (Urowitz et al., 2008) and consequent endothelial dysfunction (54.8% of SLE patients in comparison to 26.3% of control population) is frequently observed in women with SLE (El-Magadmi et al., 2004), contributing to both clinical and subclinical CVD (Kiani et al., 2011). These conditions include arterial hypertension (33% to 74%) (Sabio et al., 2011) subclinical atherosclerosis (6-fold increased risk in SLE patients compared with control population) (Kiani et al., 2011; Wu et al., 2016), carotid plaque, arterial stiffness (Parker et al., 2014) and coronary artery calcification (detected in 30% to 58% of SLE populations) (Parker et al., 2014; Kiani et al., 2015), the pathology of focus in this study. Deposition of coronary calcium is an independent predictor of both short- and long-term cardiac events (Chang et al., 2009). The increased risk of developing atherosclerosis in autoimmune diseases is suggested to be due to persistent inflammation in the vasculature involving various inflammatory mediators such as leucocytes, cytokines and chemokines, and long-term exposure to steroid therapy (López-Pedrera et al., 2010; McMahon et al., 2011). Atherosclerosis is a vascular inflammatory disorder where accumulation of lipids, inflammatory cells, platelets and vascular smooth muscle cells (VSMCs) (principal cell type in the medial layer of the arterial wall) is triggered by endothelial dysfunction, and VSMC

proliferation and migration to the intimal layer occurs (Paudel et al., 2016). There is an increase in thickness of the intima-media and carotid plaque formation in these patients, in comparison to control (Shoenfeld et al., 2005; López-Pedrera et al., 2010).

1.2 Vascular Calcification

Coronary artery calcification (CAC) refers to vascular calcification that occurs in the coronary arteries. Vascular calcification is a highly regulated, active process in which coronary artery smooth muscle cells (CASMCs) undergo apoptosis and sequential transdifferentiation into osteogenic-like cells, leading to deposition of hydroxyapatite minerals within the vessel wall (Evrard et al., 2015; Durham et al., 2018). Triggers for apoptosis includes interaction of CASMCs with inflammatory cells expressing cell surface death ligands and secretion of pro-apoptotic cytokines such as tumour necrosis factor (Iyemere et al., 2006). An *in vivo* study by Aikawa and colleagues (2007) confirms atherosclerosis-related vascular calcification to be an inflammatory disease due to the involvement of macrophage preceding osteogenic activity within the atherosclerotic plaque. Additionally, work conducted by Demer and colleagues (Watson et al., 1994; Tintut et al., 2000; Parhami et al., 2002) demonstrated cytokines such as tumour necrosis factor-α (TNF-α), interleukins-1β, -6 and -8, and transforming growth factor-β from macrophages to stimulate osteogenic differentiation and mineralisation *in vitro*.

At the cellular level, vascular calcification shares many similarities with osteogenesis, and is promoted by endothelial damage and dysfunction from inflammation as a result of oxidative stress (Fakhry et al., 2017). Inflammation can be an indicator of amplified oxidative stress and evidence to support the involvement of oxidative stress in atherosclerosis in animal studies is available (Steinberg, 2009). Chronic inflammation seen in SLE patients is indicative of elevated conditions of oxidative stress, causing vascular imbalances that can stimulate atherosclerosis formation, followed by osteoblast or chondrocyte maturation and calcium deposition (Fakhry et al., 2017). Furthermore, studies have documented that inflammation can trigger osteoblastic activity, resulting in mineralisation in the early stages of atherosclerotic plaque formation (Demer and Tintut, 2003; Aikawa et al., 2007).

Within the vasculature, the role of endothelial cells and VSMCs are crucial in maintaining vascular tone and homeostasis (Iyemere et al., 2006; Chang et al., 2014). VSMCs are known to have a plasticity function. These cells maintain normal blood pressure by cycles of contraction

and relaxation which changes vessel diameter and allow haemodynamic regulation (Chang et al., 2014; Frismantiene et al., 2018). In addition, VSMCs provide resistance to mechanical stress and structural support in the blood vessels, as well as contribute to smooth muscle and vessel repair by reversible differentiation, acquiring a reparative phenotype (Owens et al., 2004; Frismantiene et al., 2018). This vascular wall renewal or repair of injury to the vessel occurs constantly at a low rate, but this VSMC plasticity is compromised under conditions which lead to failure of the controlling mechanisms (Frismantiene et al., 2018). This increases the risk of developing atherosclerotic plaques (Owens et al., 2004). Upon progression of the disease, calcium phosphate crystals form within the intimal layer, similar to the crystals detected in bone (Iyemere et al., 2006). Subsequently, the disease is driven by a positive feedback loop of mineralisation and inflammation (Nadra et al., 2005).

VSMCs also participate in maintaining and remodelling of the extracellular matrix (ECM) of blood vessels (Durham et al., 2018). The ECM predominantly contains various types of elastin fibres and collagen, and many studies show an association between degradation of the ECM and atherosclerosis (Chen et al., 2018). Under pathological conditions VSMCs transform into osteo/chondrocytic-like cells with macrophage- and lipid-rich atherosclerotic lesions. There is a reduction in elasticity of the vessel wall which disrupts the normal haemodynamic of the vessel (Shanahan et al., 1999; Iyemere et al., 2006). This loss of arterial elasticity and stiffness has been associated with substantial cardiovascular mortality and morbidity, relating to hypertension, stroke and myocardial infarction (Manzi et al., 1997; Demer and Tintut, 2008).

Many studies and disease models have used VSMCs and vascular tissues, but the biology of these cells in poorly understood, hence why there has not been any success in treating or preventing VSMC-related conditions (Frismantiene et al., 2018). Morphological changes are observed in *in vitro* cultures of VSMCs, referred to as phenotype switching or transition, in response to different environments and treatments (Owens et al., 2004) and this includes transdifferentiation to osteogenic-like cells.

Typically, young women are at a low risk of early-onset CAC, but the opposite is seen in young women with SLE (Kiani et al., 2015) and this cannot be explained by traditional risk factors (McMahon et al., 2011). Comparatively, a study by Kao et al. (2008) found an increased prevalence of asymptomatic CAC in non-diabetic women with SLE (48%) compared to age- and

race-matched healthy controls (35%). Currently, the mechanism of this process in SLE patients is poorly understood. Regardless of its substantial clinical impact, no medical therapies exist to prevent or treat CAC. Identification of the pathways governing this disease can help in producing a novel therapeutic strategy and will be the focus of this study.

1.3 Microvesicles

Microvesicles (MV) are small membranous structures released from many cell types through either an exocytosis "budding" process (Schiro et al., 2014; Buendía et al., 2015) or an endosomal pathway via fusion of multivesicular bodies with the plasma membrane and exosome release (Krohn et al., 2016). This occurs in response to cell activation, stress or apoptosis triggered by inflammatory cytokines, cardiovascular risk factors and/or complement activation (Burger et al., 2013; Goettsch et al., 2013; McCarthy et al., 2016). During this process, the MV takes a part of the cell's contents with it, safely packaged within a specific part of the membrane, consequently the MV contains specific proteins from the parent cell but in different amounts (Majeska and Wuthier, 1975; Paudel et al., 2016). Furthermore, the stimulus that triggers MV release determines the different and distinctive role of the MV in disease (McCarthy et al., 2017). The vesicles have a metabolically active outer membrane, giving protection to its cytoplasmic components which consists of proteins, microRNA (miRNA or miR) and other components from the paternal cell (Brodsky et al., 2004; New et al., 2013; Buendía et al., 2015). The contents of a MV dictates its function and allow for participation in both physiological and pathological systems (New and Aikawa, 2013). In addition, MVs partake in the modulation of cell apoptosis and proliferation, maintaining vascular homeostasis and inflammation, which explains their involvement in the pathogenesis of immune-mediated diseases as shown in a prospective study carried out by our group (Parker et al., 2014; McCarthy et al., 2017).

MVs are able to mediate communication between cells and allow exchange of genetic information, as well as participate in adaptive immune responses (New and Aikawa, 2013). Moreover, these vesicles can regulate function of target cells, depending on the stimulus which triggered MV release, by delivering and transferring their contents to several recipient cells (Mause and Weber, 2010; Jansen et al., 2013; Pan et al., 2016). The exchange of molecules occurs as the vesicle binds to surface receptors on the target cell with endocytosis by recipient

cells and fusion with the membranes, allowing the vesicle to release its content into the target cell cytosol (Goettsch et al., 2013).

Increased endothelial cell-derived MVs (EMVs, 100nm to 1µm in diameter) have been observed in patients with traditional CVD risk factors (Preston et al., 2003; McCarthy et al., 2016), and EMVs predict adverse outcomes in patients with CVD (Sinning et al., 2011; Parker et al., 2014; Paudel et al., 2016). Evidence shows that EMVs are able to mediate intracellular signalling through their ability to transfer bioactive molecules to recipient cells, and they show paracrine and autocrine actions on vascular cells (Distler et al., 2005; McCarthy et al., 2016). In addition, previous work by our group found significantly higher levels of EMVs in patients with active and low-disease activity SLE, in relation to endothelial dysfunction, compared to controls (Parker et al., 2014; Schiro et al., 2014). EMVs are also present in healthy controls, but at low levels. Further research also demonstrated lowered cardiovascular risk as a result of reducing the number of circulating EMVs by immunosuppressive therapy (Parker et al., 2014). However, it is unclear whether the composition of MVs can directly stimulate osteogenic differentiation of CASMCs and subsequent mineralisation.

1.4 MicroRNAs

It has been established that the majority of the genome is transcribed into ribonucleic acid (RNA) and most of this transcriptome produces regulatory RNA, important in the regulation of biological processes (Mattick and Makunin, 2006; Condorelli et al., 2014). Consequently, a huge part of the genome is dedicated for the production of regulatory non-protein coding RNAs (ncRNAs), small interfering RNAs (siRNAs) and miRNAs (Mattick and Makunin, 2006). The miRNA family is known to perform a role in regulating proteins involved in the pathophysiological response to stress (Leung and Sharp, 2010). In addition, miRNAs constitute an ample ncRNA species with around 2,000 miRNAs in humans (according to miRbase; http://www.mirbase.org) coded for by sections within the introns of coding or non-coding transcripts synthesised by RNA polymerase II, outnumbering phosphates and kinases, demonstrating their influence on regulating cellular processes (Mattick and Makunin, 2006; Leung and Sharp, 2010; Goettsch et al., 2013). More than 60% of all mammalian mRNAs are predicted targets of miRNAs (Friedman et al., 2009).

miRNAs are single-stranded, non-coding RNAs, 18 to 24 nucleotides in length. They negatively regulate post-transcriptional gene expression by complementary binding to target miRNA 3'-untranslated region (3'-UTR), inhibiting translation and/or degrading the mRNA, and therefore, causing gene silencing (Wahid et al., 2010; Goettsch et al., 2013; Wojciechowska et al., 2017). Furthermore, this forms a 3-dimensional communication between miRNA and mRNA within a cell, changing in response to age and pathophysiological processes (Condorelli et al., 2014). This function allows miRNAs to act as genetic switches or fine-tuners for a diverse range of biological processes, and it has been reported that miRNA dysregulation can impair cellular function and aid in disease progression (Goettsch et al., 2013).

Typically, miRNAs are intracellular but can be released into the bloodstream in combination with proteins or encapsulated by microvesicles formed from the plasma membrane, or expelled by membrane shedding of the cell and carried to different sites where they interact with other cells (Condorelli et al., 2014; Wojciechowska et al., 2017). miRNAs have been implicated in playing an important role in the pathophysiology of CVDs, including the control of VSMC proliferation and maturation, neoangiogenesis, vasculogenesis and endothelial function (reviewed in Condorelli et al., 2014). A series of miRNAs have been demonstrated to regulate osteoblast differentiation and mineralisation (Hu et al., 2010; Fakhry et al., 2013). The potential role of miRNAs in SLE-related CAC will be investigated in this research project.

Consequently, examining the effects of over- or under-expression of miRNAs can be useful in determining the therapeutic targets for prevention and treatment of CAC. miRNA levels can be modified within *in vitro* models of calcification by two approaches: mimicking miRNA function (over-expression), or by silencing its action (under-expression) (Condorelli et al., 2014; Wojciechowska et al., 2017). miRNA expression has been shown to be inhibited using artificially designed transcript decoys called "sponges" which contain miRNA-binding sites in the 3'-UTR of non-toxic gene and can deplete the cell of free and active target miRNA (Leung and Sharp, 2010; Dangwal and Thum, 2014; Nguyen and Chang, 2017).

A study by our group on miRNA levels in patients with SLE has shown a higher level of miR-3148 compared to controls in this population. This indicated a potential role for miR-3148 in the inflammatory process associated with this disease and potentially the subsequent formation of mineralised matrix in the coronary arteries. In addition, a bioinformatics analysis

using DIANA-TarBase algorithms (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8/index) predicted that the following miRNAs are involved in the regulation of genes coding for proteins that have been previously linked to the pathways leading to VC: miR-30d-5p, miR-320a and miR-3148 (Vlachos et al., 2015). These miRNAs were then selected to be investigated in the current study.

1.5 MicroRNA Target Genes involved in Calcification

The development and maintenance of normal homeostasis of the vasculature requires accurate regulation of a series of genes associated with multiple signalling pathways, and the initiation and advancement of vascular diseases are often caused by abnormalities in signalling between these genes. Since VSMCs exhibit high phenotypic diversity, the transcriptional regulation of these cells is also very complex, and differentiation is driven by multiple transcriptions factors. Expression of multiple genes can be affected by a single miRNA (convergent miRNA pathway), and multiple miRNAs may compete or interact with each other to regulate a single mRNA with multiple miRNA binding sites in the 3'-UTR region (divergent miRNA pathway) (Condorelli et al., 2014). This explains why only a small number of targets may cause wide-ranging changes in biological responses and phenotypes (Anglicheau et al., 2010; Goettsch et al., 2013; Katano et al., 2017).

Runt-related transcription factor 2 (Runx2), also known as Cbfa1 or AML3, plays a crucial role in osteoblast differentiation and chondrocyte maturation in bone (Sun et al., 2012). It regulates gene expression of proteins involved in bone mineralisation and bone matrix proteins, including osteopontin, osteocalcin and receptor activator of nuclear factor κ-B ligand (RANKL) (Zamurovic et al., 2004; Leopold, 2014), affecting differentiation and maintenance of osteoprogenitor cells (Zhang et al., 2011). Runx2 expression in vascular cells under normal physiological conditions is typically low, but the expression is elevated in calcified vascular tissue samples from atherosclerotic plaques (Sun et al., 2012). Research shows that aberrant expression of Runx2 in CASMCs induces osteo-/chondrogenic phenotype in these cells (Speer et al., 2010). Lin and colleagues (2015) demonstrated the absence of Runx2 in VSMCs isolated from Runx2 knockout mice and they did not calcify under high-phosphate conditions *in vitro*. In addition, these Runx2 knockout mice were less susceptible to phosphate-induced calcification compared to control. Moreover, Runx2 overexpression has been acknowledged in

atherosclerotic calcified human vascular tissue specimens (Tyson et al., 2003; Aikawa et al., 2007) and in calcified aortic smooth muscle cells in mice (Steitz et al., 2001), stressing its role in CAC. These findings suggest that pathways controlling Runx2 expression are potential targets for prevention and therapy of vascular calcification.

Bone morphogenetic proteins (BMPs) are a group of growth factors which are crucial in the development of the vasculature and are involved in vascular disease (K. I. Boström, 2016). It is known that BMPs are able to activate osteogenic differentiation of cells (K. Boström et al., 1993) and have been associated with atherosclerosis. BMP2 is a pro-calcifying stimulus which upregulates intracellular levels of inorganic phosphate and triggers the expression of Runx2 (Nanoudis et al., 2017). *In vitro* and *in vivo* studies in bone tissue show that inhibition of BMP2 prevents differentiation of osteoblasts and bone formation and protects against atherosclerosis and vascular calcification in vascular tissue (Evrard et al., 2015).

Further genes that have been associated with calcification include Sirtuin 1 (Sirt), a gene associated with cell longevity and a marker for cell senescence through regulation of cell cycle, metabolism and apoptosis (Takemura et al., 2011). Sox9 (SRY [sex-determining region Y]-box 9 related transcription factor) is another gene of interest in calcification. It is a chondrogenic regulator primarily expressed in resting and proliferating chondrocytes which is known to interrupt the activity of Runx2 (Zhang et al., 2012; Loebel et al., 2015). Finally, matrix metallanoproteinase-16 (MMP16) is a part of a group of proteolytic enzymes that are able to regulate and partake in extracellular matrix remodelling and degradation. Under pathological conditions, MMPs are considered to participate in events leading to the changes in proliferation and migration of VSMC and vascular collagen and/or elastin content, potentially leading to an increase in arterial stiffness (Peeters et al., 2017).

All three miRNAs, -30d-5p, 320a and 3148, have been found to target the calcification protein regulator Runx2 and the transcription factor SOX9. Only miR-3148 was found to target BMP2, but with a very low rank (number 3501). miR-3148 and -30d-5p target Sirt1. The gene coding for MMP16 was not shown to be a target of any of the miRNAs according to the bioinformatics analysis results, but since this group of metallanoproteases have been considered to play a potential role in arterial stiffness, the changes in the level of expression of this enzyme was determined in this study also.

1.6 Gateway® Cloning Technology for use in Protein Functional Analysis

The Gateway® Cloning technology is a recombinational cloning system, an alternative to restriction enzyme cloning, and is able to produce high-throughput parallel generation of Expression Clones *in vitro* (Doyle, 2009). The system is based on the recombination between attachment sites (*att* sites); B, P, L and R. and there is no net loss or gain of nucleotides during this reaction. However, the DNA fragments flanking the recombination sites are switched resulting in *att* sites that are hybrid sequences containing nucleotides contributed by each parental vector, maintaining the reading frame register (Hartley et al., 2000). The target genes are flanked by two slightly different *att* sites and are unable to combine with each other, allowing the recombination reaction to occur while retaining the reading frame (Invitrogen, 2003; Doyle, 2009).

In this project, a Destination Vector (DV) with a lentiviral vector backbone was generated via the BP recombination reaction where the *att*B sites of the Expression Clones and *att*P sites of the donor vector recombine. The DV contains a Gateway® "cassette", replacing the target gene, which encodes elements needed to express the gene of interest in the suitable system (i.e. *E. coli*), including resistance markers and promoters; chloramphenicol resistance gene and *ccd*B suicide gene (Doyle, 2009). The *ccd*B gene encodes CcdB protein which interferes with *E. coli* DNA gyrase, resulting in inhibition of the growth of these cells, making it a toxic gene (Bernard and Couturier, 1992). The consequential *att*R sites of the DV are able to recombine with the *att*L sites of Entry Clones in an LR reaction to produce an Expression Clone (as portrayed in Figure 1) (Invitrogen, 2003; Doyle, 2009).

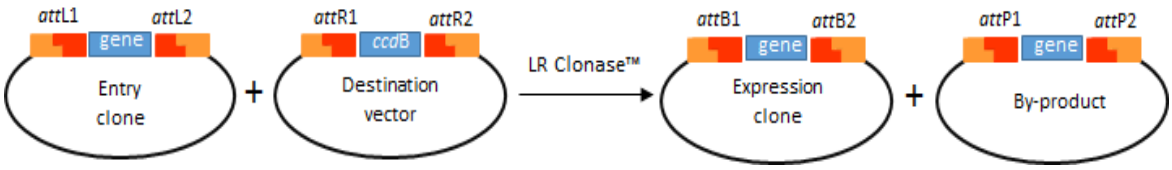

Figure 1 *A diagram to represent the LR reaction in a Gateway Cloning System*

During the LR reaction, LR clonase is required; it facilitates the recombination of the *att*L sites of the Entry Clone with *att*R sites of the destination vector to create an Expression Clone with *att*B sites.

Consequently, a strain of *ccd*B resistant *E. coli* is required to promote growth of bacteria containing the DV and to eliminate non-recombinant vectors. The DV will then be recombined with an Entry Clone vector containing the gene of interest via the LR reaction, producing the Expression Clone containing the DV lentiviral backbone.

The purpose behind constructing a DV of this nature is that it not only fulfils the requirement for this study, investigating the effects of the specific miRs selected, but will enable new lentiviral vectors to be produced by our group with ease and speed in the following three different ways:

1. A plasmid with the gene of interest flanked by *att*L1 and *att*L2 sites and Kanamycin resistance could be purchased:

   The plasmid acts as the Entry Clone and is mixed with the DV (containing the suicide gene *ccd*B) in the presence of LR Clonase™ (Figure 1). Then clones would be selected for ampicillin resistance to isolate the putative lentiviral Expression Clone.
2. PCR could be performed on the gene of interest with *att*B1 and *att*B2 sites added to primers:

   The PCR product is combined with donor vector pDONR™221 and incubated at 25°C for up to 24 hours. The DV is then added and selected for ampicillin resistance after incubation at 25°C for up to 24 hours.
3. A plasmid with the gene of interest flanked by *att*B1 and *att*B2 sites and ampicillin and kanamycin resistance could be purchased:

   Perform BP recombination reaction with pDONR™221 and the purchased plasmid and select for kanamycin resistance from an overnight growth of bacterial culture. Extract DNA from a colony and perform the LR reaction with the DV, followed by selection for ampicillin resistance.

1.7 Hypothesis

This study tests the hypothesis that miR-3148 released from EMVs protects against the osteogenic differentiation of human CASMCs *in vitro* by targeting Runx2 gene.

1.8 Aims and Objectives

The aim of this study was to validate the association of miR-3148 with EMV-driven and SLE-related coronary artery calcification and explore the function of these possible regulators in disease progression. The effect of miR-3148 in the process of β-glycerophosphate-induced osteogenic differentiation of CASMCs was also evaluated. In addition, this study investigated the effects of altered levels of the following: miR-30d-5p, miR-320a and miR-3148, on genes involved in CAC in CASMCs. A DV was also be generated as a part of the Gateway® Cloning system to make cloning of lentiviral vectors in subsequent studies in the future effortless.

2 Methodology

2.1 Cell Culture and *in vitro* Calcification of Human Coronary Artery Smooth Muscle Cells

Early Passage Human CASMCs (Caltag Medsystems, ZHC-3311) were used as the most appropriate cell of choice for this study as CASMCs are prone to calcification in cardiovascular disease patients and are widely used as an *in vitro* calcification model, as previously described by our group (Liu et al., 2011; Yan et al., 2011; Bartoli-Leonard et al., 2019). These cells were cultured in Smooth Muscle Cell Growth Medium 2 (SMC growth medium) (Promocell, C-22262), supplemented with 5% Foetal Calf Serum (FCS), 2ng/ml basic fibroblast growth factor, 5µg/ml insulin and 0.5ng/ml epidermal growth factor (Promocell, C-39262) in T75 cell culture flasks (ThermoFisher Scientific) at 37°C, 5% CO_2, with media change twice a week. CASMCs used for this study were from the 5^{th} to 8^{th} passage.

Early Passage Human Embryonic Kidney (HEK) 293T cells were used for transfection studies as they are the most widely accepted cell line for recombinant DNA technology and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, BE12-614F), 10% FCS, 1% L-Glutamine (Corning, 15393631) and 1% Penicillin-Streptomycin (Lonza, 17-603E) in T75 cell culture flasks at 37°C, 5% CO_2, and this was used as the regular culture medium. HEK 293T cells used for this study were from the 15^{th} to 21^{st} passage.

At confluence (as shown in Figure 2), 8.7 x 10^6 cells in T75 flasks, the adhering cell monolayer was washed with 10 ml Phosphate Buffered Saline (PBS) (Lonza, BE17-512F) and then 2.5-3 ml of Trypsin EDTA (Lonza, BE17-161E) was added to dislodge the cells, all at 37°C. 5 ml of fresh

regular DMEM medium was then added to the cells to block the action of trypsin, and the cell suspension was centrifuged at 300 x g for 5 minutes, 21°C. The pellet was re-suspended in T75 flasks in regular DMEM (for 293Ts) for future calcification experiments or in SMC growth medium (for CASMCs) for cell maintenance at a density of 4 x 10^5 cells/cm^2, and incubated overnight at 37°C. High glucose DMEM was used for all experiments.

For the calcification assay, 5 mmol/L β-glycerophosphate (βGP) and 2.6 mmol/L $CaCl_2$ in DMEM (4.5 g/L glucose) was used to induce mineralisation of the cells which occurred at 15 to 18 days. CASMCs were seeded in SMC growth media in 6-well plates (Nunclon™ Delta Surface, ThermoFisher Scientific) at a density of 1.2 x 10^6 cells per well. The aim was to perform all experiments with technical duplicates and biological triplicates so that there would be at least *n = 3* for statistical purposes. However, due to lack of time within this one-year study, and the challenges associated with growing human primary patient cells, this was not always possible. Therefore, *n* numbers are provided in each figure legend and statistical analysis was carried out when appropriate. Conclusions were not drawn when insufficient experiments were performed, but an inference could be made and this is taken into account in the discussion.

Cells were treated with 500 µl lentiviral vector containing genes for the following miRNAs and their sponges: miR-30d-5p, miR-320a and miR-3148, and plasmid pLL3.7 used as the control, in osteogenic media. Media change on cells occurred twice a week for 18 days, or until the presence of calcific nodules (Liu et al., 2011).

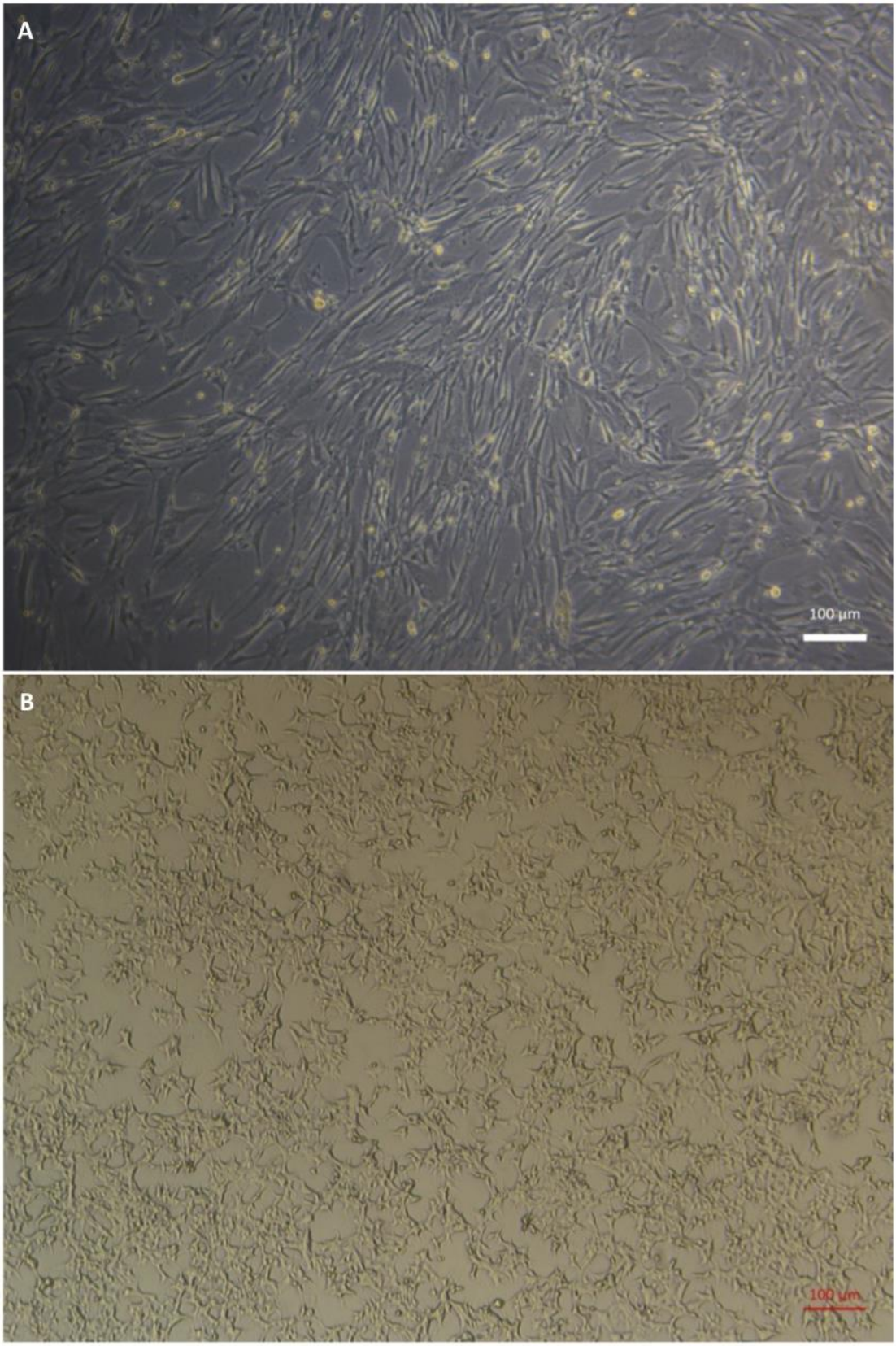

Figure 2 *Cells at confluence*

Cells were maintained in T75 flasks and transferred to T75/T175 flasks or 6-well plates. **(A)** Passage 3 Human Coronary Artery Smooth Muscle Cells at day 4 **(B)** Passage 5 Human Embryonic Kidney 293T cells at day 4.

2.2 Alizarin Red Staining and Quantification to Measure Mineralised Matrix Formation

Alizarin Red S is a well established staining procedure to identify the presence and extent of calcium deposition in cultures of VSMCs. At 15-18 days, when calcific nodules were observed, media was aspirated from CASMCs and the cells were washed three times with PBS (1 ml/well) then fixed in 4% paraformaldehyde (700 µl/well) (Sigma-Aldrich, P6148) for 10 minutes at room temperature (RT). The cells were re-washed and stained with 1 ml 2% Alizarin Red S (ARS) (pH 4.2 with hydrochloric acid, Sigma-Aldrich, F5533) for 5 minutes at RT. The cells were washed with distilled water four times. To quantify mineralisation, the ARS stain was eluted using 1 ml of 10% formic acid (Sigma-Aldrich, F0507) for 5 minutes on a rocker and 200 µl of elution added in triplicate to a 96-well plate. Absorbance was measured at 414 nm (Liu et al., 2011).

2.3 Inflammation assay and Isolation of Microvesicles, miRNA Extraction, Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR)

CASMCs were seeded in 6-well plates and treated after 24 hours with FCS-free DMEM (2 ml/well), and at 48 hours with TNF-α (1 µl/ml) in DMEM (10% exosome free FCS). After 24 hours of treatment with TNF-α the media was aspirated and centrifuged at 1,500 x g for 10 minutes. The supernatant was then ultracentrifuged at 100,000 x g for 60 minutes (at 4°C) to isolate microvesicles. miRNA was extracted from the microvesicles and cells using a miRNA isolation miRNEASY kit (Qiagen, 217004) as per the manufacturer's instructions. miRNA was reverse transcribed using miRCURY LNA RT kit (Qiagen, 339340) using SureCycler 8800 at 42°C for 60 minutes, followed by 95°C for 5 minutes. qPCR was conducted using miRCURY LNA miRNA PCR assay kit (Qiagen, 339306) as follows; 10 seconds at 95°C and 1 minute at 56°C for 40 cycles. The primers for miR-3148 (U G G A A A A A A C U G G U G U G U G C U U) and Let-7a-5p miRNA (U G A G G U A G U A G G U U G U A U A G U U) were used. All PCR amplifications were carried out using StepOnePlus system (Applied Biosystems). Quantification was performed using the $2^{-\Delta\Delta Ct}$ method.

2.4 Vector Preparation: Electrophoresis and Plasmid Cloning by Restriction Enzyme Digest

The online database National Centre for Biotechnology Information (NCBI) was used to produce the sequences for each miRNA (as shown in Table 1).

Table 1 *Sequences of artificially designed transcripts coding for the miRNAs*

The sequences for each miRNA are highlighted in "bold" and the flanking regions are 100bp of the sequence surrounding the gene in the genome.

DNA	Sequence	NCBI Gene ID
hsa-miR-30d-5p	TCTATTGTTCAGCACTAGAAATTATATAAATTATTAG CTGAAGATGATGACTGGCAACATTTATGTCTGTTCCT CCTCTTAAATTTCTTGTTCAGAAAGTCT**GTTGTTGTAA ACATCCCCGACTGGAAGCTGTAAGACACAGCTAAGC TTTCAGTCAGATGTTTGCTGCTAC**CGGCTATTCACAG ACATCCTCTTGATATAATTCTGTCCCGGAGTGGAGTT GAGGAGGCTATAAAATGTGTGGGAAAACCTCAGAAA TCTTTAGCTGCATTCTCGAGG	407033
hsa-miR-320a	TCGGCGGAAGTCTGCGTGGCAGGGCCTGGGCGCCGC CATCTTGGCGCGGGGCGGAAGTGACGTTAGGGGGGC GGGACTGGGCCACAGTATTTATCAGGCGGC**GCTTCG CTCCCCTCCGCCTTCTCTTCCCGGTTCTTCCCGGAGTC GGGAAAAGCTGGGTTGAGAGGGCGAAAAAGGATGA GGT**GACTGGTCTGGGCTACGCTATGCTGCGGCGCTC GGGGGTCTTGGCCTCCGGGCGGTGGCGTGGAGGCGC CAAGATCAGGGTCCCGGGTTTTGTCGGCCACCTCGAG G	407037
hsa-miR-3148	TCTCTTCCCACAGGAAGGCAATGTGTGGAATGGAGA AGGCTGCTTCAGCAACATCCTCTCCCATGCACCTTAA ACGTCTCTTTCTTTCTCAGCTCCTTTCTG**GAGTTAAGA TGGAAAAAACTGGTGTGTGCTTATTGATGTAGCCAA CAAGCATACATCAGTTTTTTCCAACTTAACTC**CAGTA TTTTCCCCAATTCATCCTGAAATTGCTGCCCTATCCAT TCTCCCTCCTACACAGCCAAGATTCTTAAAAAACCAA TCCAAATTTGCAGAATCTCCG	100422876

A web-based tool, miRNA sponge generator and tester (miRNAsong, http://www.med.muni.cz/histology/miRNAsong) was used to produce the sponge sequences for each miRNA (Barta et al., 2016). Table 2 shows the criteria used to produce the sequence for each miRNA sponge.

Table 2 *The miRNA sponge sequence constructs that were purchased*

miRNAsong was used to make miRNA sponges specific to each miRNA to reduce the levels of miRNA in the calcification model.

Sponge Sequence Criteria	**Sponge Sequence**
T*he miR-30d-5p sponge sequence included 3 miRNA-binding sites (MBS), a bulge at nucleotide position 11-14, and a spacer sequence AAAAU between individual MBSs. Test for off-targets showed that the generated sequence binds to miR-30a-5p and miR-30e-5p as well as miR-30d-5p (free energy -86.4 kcal/mol) (settings: -25 kcal/mol cut off and canonical 6-mer seed).*	C T T C C A G T A T T G A T G T T T A C A A A A A T C T T C C A G T A T T G A T G T T T A C A A A A A T C T T C C A G T A T T G A T G T T T A C A
The miR-320a sponge sequence included 3 miRNA-binding sites (MBS), no bulge, and a spacer sequence AAAAAA between individual MBSs. Test for off-targets showed that the generated sequence binds only to 39 other miRs as well as miR-320a (free energy -146 kcal/mol) (settings: -25kcal/mol cut off and canonical 6-mer seed).	T C G C C C T C T C A A C C C A G C T T T T A A A A A T C G C C C T C T C A A C C C A G C T T T T A A A A A A T C G C C C T C T C A A C C C A G C T T T T T
The miR-3148 sponge sequence included 3 miRNA-binding sites (MBS), no bulge, and a spacer sequence UAAA between individual MBSs. Test for off-targets showed that the generated sequence binds only miR-3148 (free energy -125.7 kcal/mol) (settings: -25kcal/mol cut off and canonical 6-mer seed).	A A G C A C A C A C C A G T T T T T T C C A T A A A A A G C A C A C A C C A G T T T T T T C C A T A A A A A G C A C A C A C C A G T T T T T T C C A

All constructs were produced to include sites for HpaI [G T T A A C] *att*B1 [C A A G T T T G T A C A A A A A A G C A G G C T], *att*B2 [A C C C A G C T T T C T T G T A C A A A G T G G T] and XhoI [C T C G A G], in the following order: HpaI – *att*B1 – target DNA – *att*B2 – XhoI. The HpaI and XhoI sites were included for the purpose of cloning by restriction enzyme digest and ligation

with lentiviral vector, pLL3.7, and the *att*B sites were included for later use in production of a DV by a BP recombination reaction as a part of the Gateway® Cloning Technology (Invitrogen).

800 ng of plasmid constructs containing the DNA sequence for the miRNAs and their respective sponges, and the lentiviral vector pLL3.7 (shown in Figure 3) (Rubinson et al., 2003) were digested using restriction enzymes for HpaI (1 µl) (NED, R0746S) and XhoI (1 µl) (NEB, R0105S), using 10x cut smart buffer (3 µl) (NEB, B7204S) in analytical grade water (to make up 30 µl final volume). pLL3.7 is a third-generation lentiviral vector containing the green fluorescent protein (GFP) reporter gene.

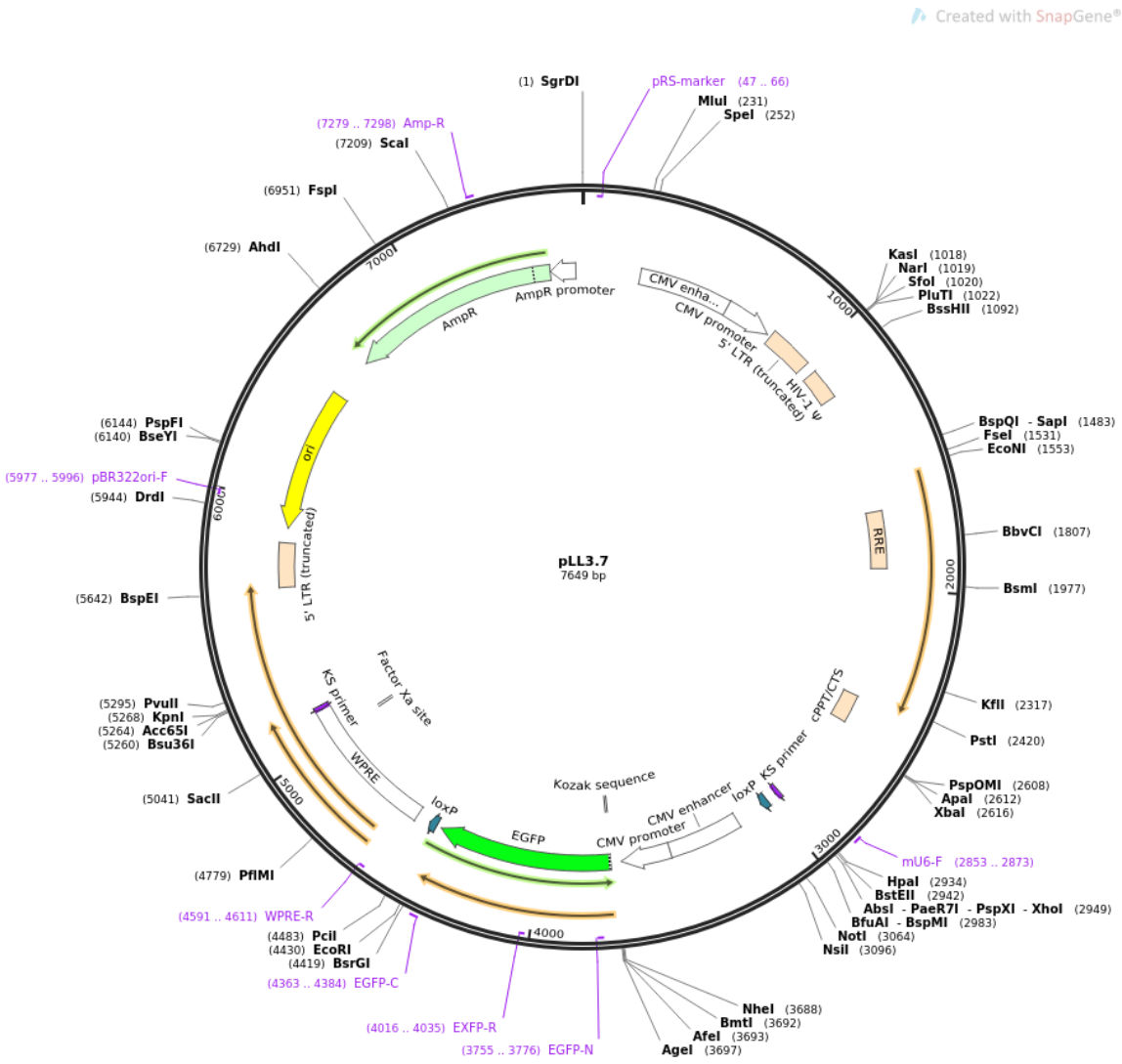

Figure 3 *Full sequence map for pLL3.7 vector. (Source: Addgene, Plasmid#11795)*

1% Agarose (Invitrogen, 165000-100) gel made in 1x TAE buffer was used for electrophoresis, using Midori Green (Nippon, MG04) to stain the DNA strands. 30 µl of each plasmid sample was loaded into the wells and electrophoresis conducted at 120 V for 30 minutes. LI-COR Image Studio software was used to capture all electrophoresis images in this study (Figure 4). The gel was incubated in 0.1 µl/ml of SYBR™ Gold (Invitrogen, S11494) in 1x TAE buffer, on a rocker at RT for 30 minutes. Bands were observed under an Ultraviolet light (UV) transilluminator and the target fragments excised using a scalpel before isolating the DNA using Zymoclean™ Gel DNA Recovery Kit (Zymo Research), eluting in 10 µl DNA elution buffer (Demeke et al., 2009). The DNA was stored at -80°C until further use.

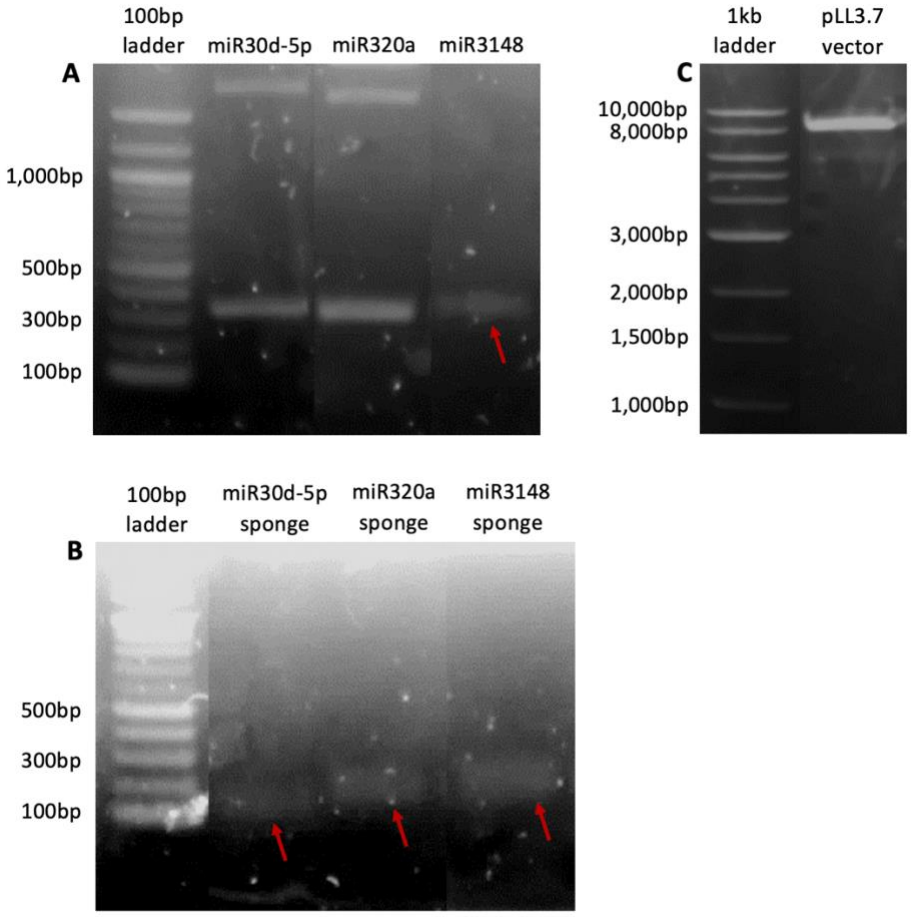

Figure 4 *Electrophoresis of digest of commercially produced DNA constructs*

Electrophoresis was carried out at 120V for 30 minutes. **(A)** The images show bands for the restriction enzyme digests of miRNA-30d-5p (329 bp), miRNA-320a (341 bp) and miRNA-3148 (330 bp) constructs. **(B)** Restriction digests of the sponges for miRNA-30d-5p (132 bp), miRNA-320a (131 bp) and miRNA-3148 (133 bp) constructs. **(C)** Restriction enzyme digest of PLL 3.7 shows only one band at 7650bp. Red arrows indicate presence of bands.

A Qubit™ dsDNA HS Assay Kit (Invitrogen, Q32854) and Qubit™ 4 Fluorometer was used to determine the concentration of DNA recovered (Mardis and McCombie, 2017). The amount of insert DNA (miRNA or miRNA sponge DNA) required for ligation (with lentiviral vector pLL3.7) was then calculated using the equation $\frac{\text{amount of vector (ng)} \; x \; ratio \; x \; insert \; size \; (bp)}{\text{vector size (bp)}}$, where the amount of vector was 30.4 ng and the vector size was 7650 bp. Ligation was carried out with a vector to insert ratio of 1:5, using 10 µl of 2 x Quick Ligase Reaction Buffer (BioLabs, B2200S), 30.4 ng vector, 1 µl Quick Ligase (BioLabs, M2200L), and analytical grade water to make up a final volume of 20 µl, followed by incubation at RT for 10 minutes.

The ligation reactions (5 µl per reaction) were transformed into One Shot™ Stbl3™ chemically competent *E. Coli* cells (Invitrogen, C737303) according to the manufacturer's instructions, followed by addition of RT S.O.C. Medium (250 µl) (Invitrogen, 15544-034). The cells were placed horizontally in an incubator with a shaker at 37°C for 1 hour (80 rpm), followed by pouring onto individual pre-warmed selective plates (3.2%, LB Lennox L agarose gel, Invitrogen, 00602061) with 25 µg/ml ampicillin, using aseptic techniques. The plates were inverted and incubated at 37°C overnight.

Individual colonies were selected from each plate and cultured in 5 ml 2% LB broth base solution (Lennox L broth, Invitrogen, 00602271), supplemented with 25 µg/ml ampicillin, and incubated overnight at 37°C on a shaker. Glycerol stocks of each culture were produced (50%, Fisher Scientific, BP229-1) and stored at -20°C. Plasmids from the remaining bacterial solution were extracted using QIAprep Spin Miniprep Kit. DNA concentration and purity was measured by UV absorbance at 260 and 280 nm using a NanoDrop™ spectrophotometer (A260/280 ratio of approximately 1.8). DNA integrity was determined by digesting 800 ng of DNA using restriction enzymes XhoI and HpaI, followed by electrophoresis of the samples (Figure 5).

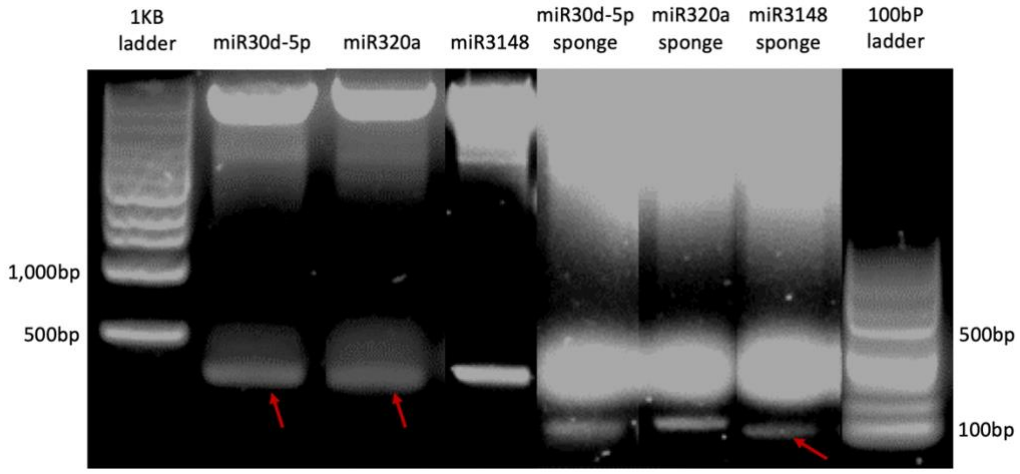

Figure 5 *Electrophoresis of digested clones of miRNA and their sponges from the mini prep*

Electrophoresis was carried out at 120V for 30 minutes. Bands in the images show presence of the insert DNA sequences: miRNA-30d-5p (329 bp), miRNA-320a (341 bp), miRNA-3148 (330 bp), miRNA-30d-5p sponge (132 bp), miRNA-320a sponge (131 bp) and miRNA-3148 sponge (133 bp). Red arrows indicate presence of bands of the expected sizes.

Plasmids and transfer vector were further amplified from 20 µl of the glycerol stocks, cultured in 20 ml 2% broth base solution (supplemented with 25 µg/ml ampicillin). DNA was purified using Plasmid Midi Kit (Qiagen, 12143) according to the manufacturer's instructions. Electrophoresis was performed at 120V for 30 minutes to determine DNA integrity (Figure 6).

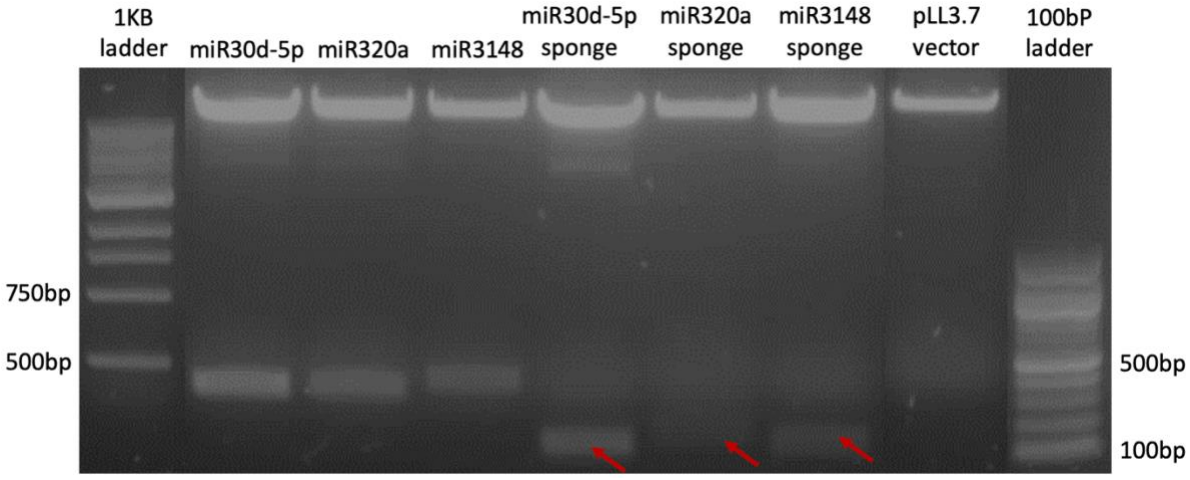

Figure 6 *Electrophoresis of digested clones of miRNA and their sponges from the midi prep*

Electrophoresis was carried out at 120V for 30 minutes in 1% agarose gel. The bands represent the insert DNA sequences: miRNA-30d-5p (329 bp), miRNA-320a (341 bp), miRNA-3148 (330 bp), miRNA-30d-5p sponge (132 bp), miRNA-320a sponge (131 bp) and miRNA-3148 sponge (133 bp). Red arrows indicate presence of bands.

2.5 Overexpression and Depletion of miRNAs in Cultured Human CASMCs

Due to the high transfectability and adaptability of HEK 293 cells, they are extensively used for lentiviral vector production (Segura et al., 2007), with the variant 293T being efficient in high-titre lentiviral production (Tiscornia et al., 2006). The enveloped protein plasmid pMD.G2 that codes for the vesicular stomatitis virus (VSV-G) envelope glycoprotein and the third-generation packaging plasmid pCMV-dR8.91 (Delta 8.9, containing HIV *gag*/*pol*/*rev* coding sequences) provided the helper functions for lentivirus production.

293T cells were seeded overnight in T175 flasks (60-70% confluency) and transfected using target DNA, pMD.G2 and pCMV-dR8.91, at a ratio of 3:1:1, respectively. Plasmids were pre-complexed with 7mM Polyethylenimine reagent (PEI, Polysciences) diluted in 150 mM NaCl. Transfection complexes (DNA-PEI) were prepared by adding 1 ml PEI mixture to plasmid DNA diluted in 150 mM NaCl (to form a final volume of 1 ml). The mixture was vortexed and incubated at RT for 10 minutes before adding to the cultured cells. At 24 hours the media was discarded and replaced. All viruses were harvested at 48 and 72 hours by centrifugation at 2000 x g for 10 minutes and the supernatant collected, filter sterilised (0.22 µM) and stored at -80°C.

CASMCs were plated in 6-well plates in SMC growth medium 24 hours prior to infection, at a cell density of 1.2 x 10^6 per well. 500 µl supernatant was added per well with 1.5 ml DMEM. GFP gene expression was visualised using fluorescent microscopy.

2.6 RNA Extraction and qRT-PCR

To test for primer efficiency, RNA from 293T cells and CASMCs, in DMEM only, was extracted using the Bioline Isolate II RNA mini kit (Ambion, 15596026) according to the manufacturer's instructions, and concentration was determined using NanoDrop Spectrophotometer at an A260/A280 absorbance ratio of approximately 2.0. For reverse transcription, 2 µg of RNA was used to produce cDNA with the Tetro cDNA Synthesis Kit (Bioline, BIO-654043), using random hexomers, as per the manufacturer's instructions. Efficacy of primers were tested in triplicate using SensiFAST SYBR Lo-ROX Mix, 2x (Bioline, BIO-94005), and real time q-PCR was run, over three 10-fold serial dilutions as follows: 95°C for 5 seconds, 62°C for 10 seconds and 72°C for 18 seconds, for 40 cycles. Primer efficiency was calculated using the equation $E = 10^{(\frac{-1}{slope})} -$

1, using the mean CT value for each dilution. Primers with amplification efficiencies ranging from 90% to 110% were used. The melting curve for each primer was also observed (Figure 7). The primers used for the current research study are summarised in

Table 3.

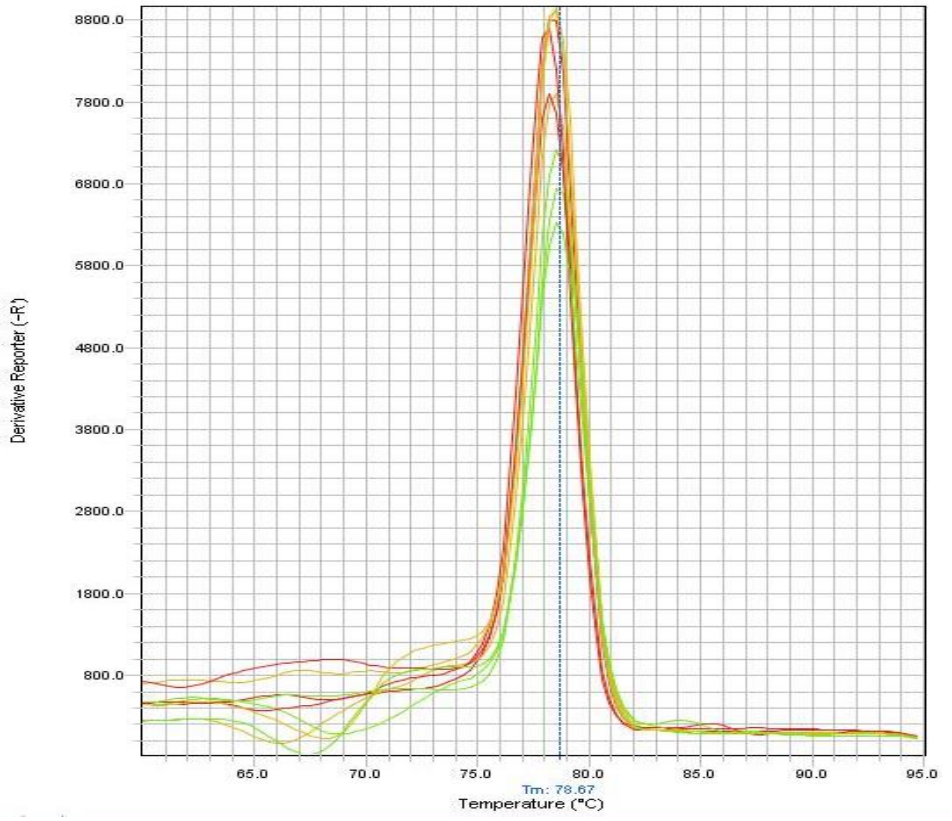

Figure 7 *Melt curve for Sirt1 primers*

This melt curve is produced from 3 different dilutions of the primers in the same RNA sample from 293T cells (passage 5) grown in high glucose DMEM.

For gene analysis, RNA from CASMCs were isolated on day 4 of treatment with osteogenic media. The expression of target genes were normalised to the expression of GAPDH and

expression levels of the genes were calculated with the comparative cycle threshold (C_T) method.

Table 3 *Primers used in this research*

Primers were designed using NCBI/Primer-BLAST (Ye et al., 2012) with the following conditions: 58 to 62°C melting temperature, maximum intron length 300, primers must span exon-exon junction.

Primer Name	**Primer Sequence (5' to 3')**
MMP16	Forward: T G C G G A A C G G A G C A G T A T T T Reverse: G G G G C T T C T T C A T C C A G T C A A T
SOX9	Forward: G C T C T G G A G A C T T C T G A A C G A Reverse: C C G T T C T T C A C C G A C T T C C T
SIRT1	Forward: A G G C C A C G G A T A C G T C C A T A Reverse: G T G G A G G T A T T G T T T C C G G C
BMP2	Forward: G G A A C G G A C A T T C G G T C C T T Reverse: C A C C A T G G T C G A C C T T T A G G A
RUNX2	Forward: G A G G G C A C A A G T T C T A T C T G Reverse: C G C T C C G G C C C A C A A A T C T C
GAPDH	Forward: C C A C C C A T G G C A A A T T C C A T G Reverse: T C T A G A C G G C A G G T C A G G T C C A C C

2.7 Alkaline Phosphatase Activity Assay

The enzyme alkaline phosphatase is a well-established early marker of osteogenic differentiation of SMCs and was used in this study to assess effects on the process following treatment with miRs. CASMCs were lysed at day 4 and day 7 with 0.05% Triton X-100 (200 µl/well) after washing with PBS thrice and freeze-thawed twice. The suspension was centrifuged at 12,000 x g for 10 minutes and the supernatants collected. Pierce BCA™ Protein Assay (ThermoFisher Scientific, 23227) was performed and the protein standard curve

produced was used to determine sample protein concentration. For the Alkaline Phosphate (ALP) activity assay, 1 mM p-Nitrophenol (Sigma-Aldrich, N7660) was diluted using 0.05% Triton to produce standards of different concentration. Standards and 20 µl of extracted protein were loaded in triplicates into a 96-well plate followed by addition of p-Nitrophenyl phosphate substrate system (180 µl/well, Sigma-Aldrich, N7653). After incubation at 37°C for 30 minutes, activity was measured spectrophotometrically at 405nm and calculated using the standard curve, as nM p-nitrophenol converted/ug protein for each lysate sample.

2.8 Protein Extraction, and Western Blot Analysis

To extract protein from CASMCs in osteogenic media at day 4, cells were treated with 30 µl RIPA lysis buffer containing 1% Phosphatase Inhibitor (Sigma-Aldrich, P5726) and 1% Protease Inhibitor (Sigma-Aldrich, P8340). The suspension was incubated on ice for 20 minutes, with vortexing every 2 minutes, followed by centrifugation for 4 minutes at 21,130 x g, at 4°C. The supernatant was removed and BCA™ protein assay kit was used to quantify protein. Loading dye (NuPAGE, Thermofisher Scientific, NP0008) was added to 15 µg protein to make up 25 µl and the proteins were electrophoresed on 4-12% Bis-Tris Gel (Invitrogen, NuPAGE™, NP0321PK2) for 2 hours at 120 V using 1x Running Buffer (Thermo Fisher Scientific, NP000102) after denaturing at 99°C for 2 minutes. The proteins were then electrotransferred to a P 0.45 PVDF Blotting Membrane (GE Healthcare Life sciences, Amersham™ Hybond™, 10600023) over 1 hour at 35V using Transfer Buffer (Thermo Fisher Scientific, NP00061). The membrane was blocked for 1 hour in blocking buffer (5% milk in 0.05% PBS-Tween). The membrane was probed in blocking buffer with anti-Runx2 antibody (dilution 1:1000, Abcam, AB76956), with anti-α-tubulin (dilution 1:5000, Abcam, AB7291) used as a loading control, overnight, at 4°C, followed by washing with 0.1% PBS-Tween and incubation with corresponding horse radish peroxidase-labelled secondary antibodies (dilution 1:2000) for 1 hour at RT. Secondary anti-mouse antibody (Sigma Aldrich, A9044) was used for both Runx2 (57 kDa) and α-tubulin (50 kDa). After washing with PBS-tween, Western ECL substrate (Millipore Immobilon® Western, WBK150500) was added to the membranes and images captured.

2.9 Gateway® Cloning Technology

Using the previously produced lentiviral backbone Expression Clone with commercially produced DNA, expressing genes HpaI – *att*B1 – target gene – *att*B2 – XhoI and ampicillin

resistance, a lentiviral backbone DV with HpaI – *att*B1 – chloramphenicol resistance ($CM^{R)}$ – *att*B2 – XhoI and ampicillin resistance sites was created via the BP recombination reaction. pDONR™221 (plasmid map displayed in Figure 8) (Invitrogen, 12536017) was digested with the following restriction enzymes; EcoRV-HF (NEB, R3195S), NSiI-HF (NEB, R3127S) and HpaI, diluted in cut smart buffer and analytical grade water. After incubation at 37°C for 2 hours, the digest was electrophoresed (Figure 9) and target DNA fragment (2.5 kb) excised followed by DNA isolation from the gel. Figure 10 outlines schematically the reaction taken place to produce the destination vector.

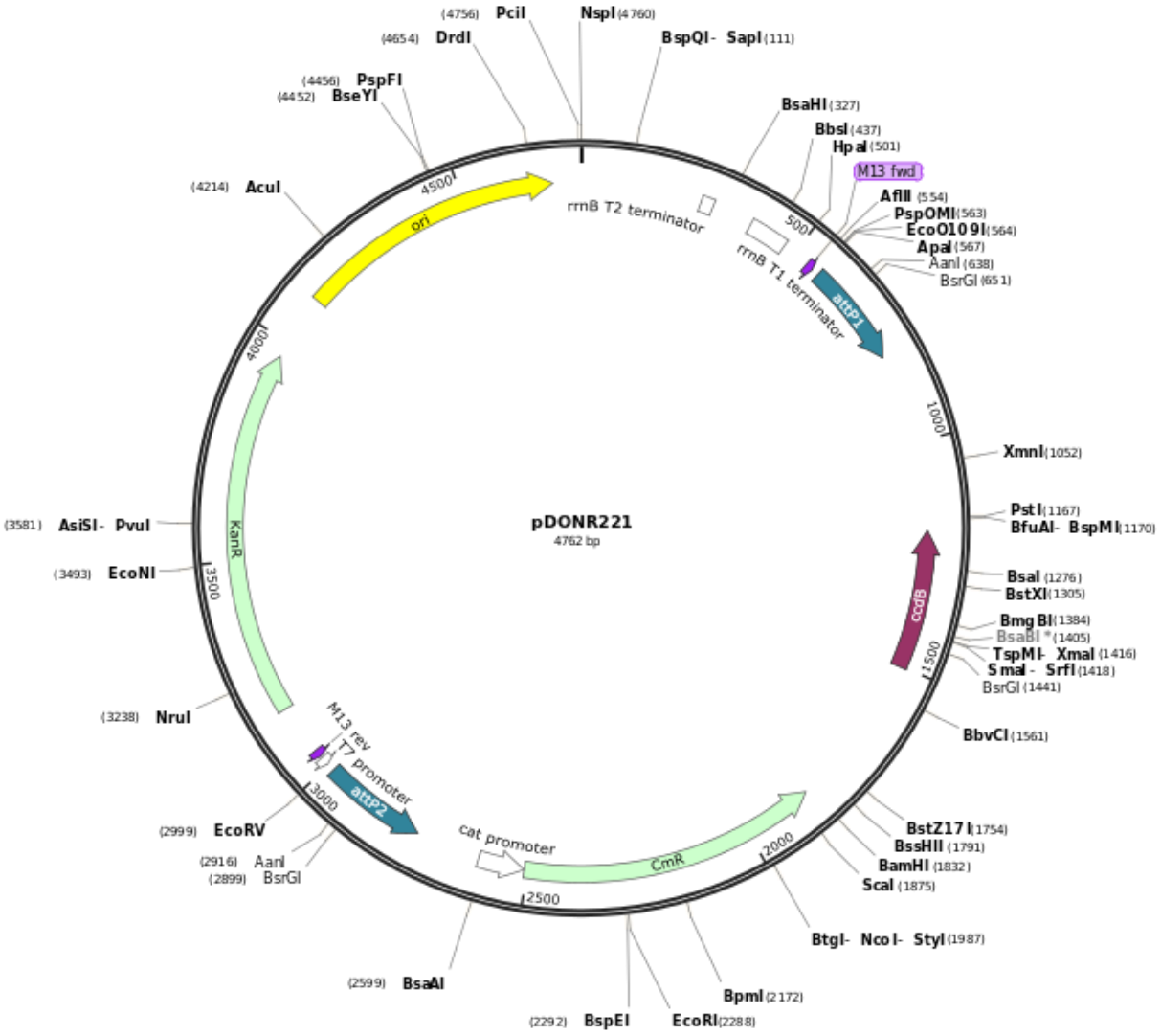

Figure 8 *Full sequence map of donor vector pDONR™221 (Source: Snapgene, pDONR™221)*

pDONR™221 contains the gene for *ccd*B and Chloramphenical resistance (CM^{R}) to allow for negative selection of the Destination Vector.

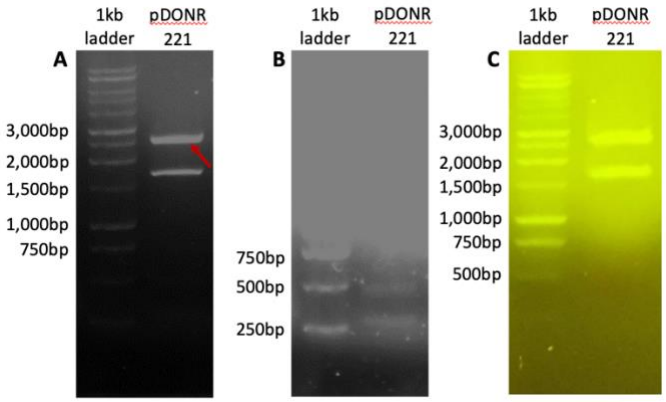

Figure 9 *Electrophoresis of digested pDONR™221*

pDONR™221 vector was digested with restriction enzymes HpaI (501 bp), EcoRV (2999 bp) and NSiI (3431 bp and 3697 bp). The digest was electrophoresed using 1% agarose gel for 40 minutes at 120V. The band at 2498 bp represents the target DNA containing the following sites; *att*P1 – *ccd*B – CM^R – *att*P, and the band at 1566 bp **(A)**, 432 bp and 266 bp **(B)** were discarded. The bands were observed under a UK transilluminator for excision **(C)** *ccd*B = Toxin CcdB gene, CM^R = Chloramphenicol resistance. The red arrow indicates target DNA.

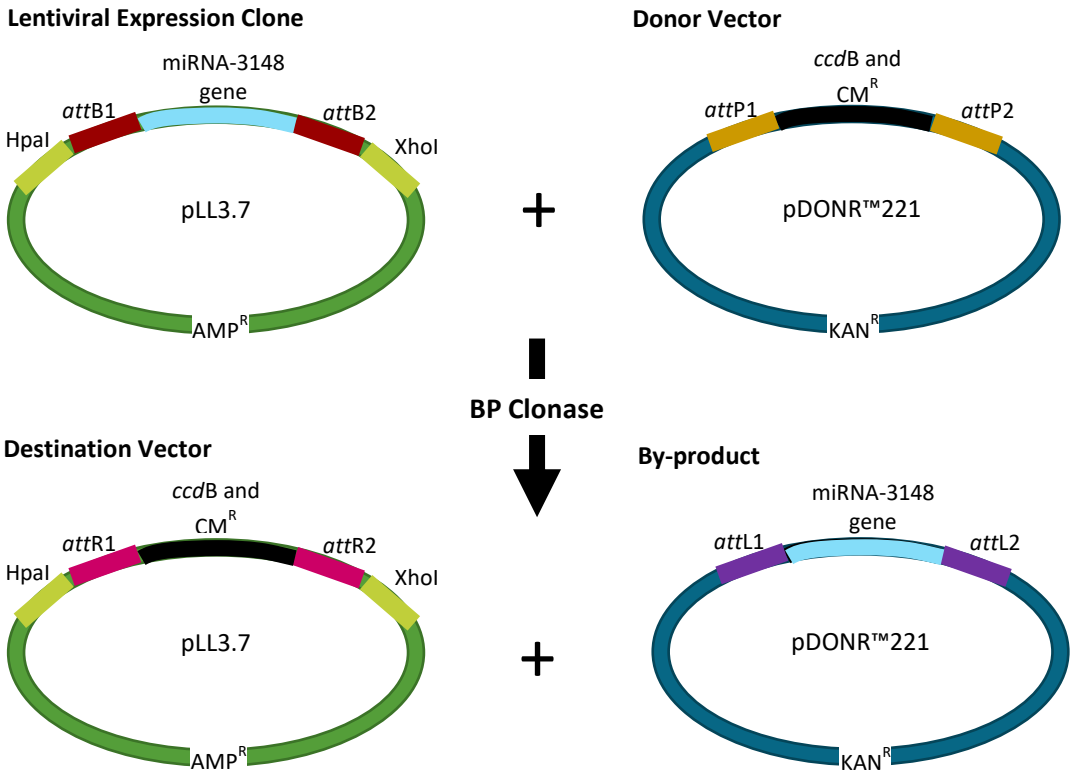

Figure 10 *A diagram to represent the BP recombination reaction to produce*

Recombination occurs between the attachment (*att*) sites of the Expression Clone and donor vector in the presence of BP Clonase™ Enzyme mix. Recombination between *att*B (25 bp) and *att*P (200 bp) sites produce *att*L (100 bp) and *att*R (125 p) sites. AMP^R = Ampicillin resistance, CM^R = Chloramphenicol resistance, KAN^R = Kanamycin resistance.

2.10 Sequencing of DNA

DNA integrity was checked by sequencing each of the DNA fragments used in this project miRNA-30d-5p, miRNA-320a, miRNA-3148, sponges for each of the miRNAs and the DV produced by the BP recombination reaction. For this sequencing, the following primer was used: 5'A G G A A A C T C A C C C T A A C T G T A A A G. 1 µl of the primer (40 µM) was mixed with 300 ng of each DNA in separate tubes and analytical grade water added to make a final volume of 10 µl. These mixtures were outsourced for sequencing by a collaborative team at the Manchester Regenerative Medicine Network (MaRM) at the University of Manchester.

2.11 Statistical Analysis

Data were found to be not normally distributed according to D'Agostino & Pearson omnibus normality test and were compared using one-way analysis of variance (ANOVA) Kruskal-Wallis test followed by Dunn's corrections. GraphPhad Prism, version 5.04, was used to analyse data. Mean and standard deviation were calculated based on three or more measurements and presented as a mean ± standard error mean (SEM). A probability (*P*) value of less than 0.05 was considered as statistically significant.

3 Results

3.1 miR-3148 Levels Increase under Inflammatory Conditions

Following the miR expression profiling in serum from patients with SLE using computational tools, the next series of experiments focused on some of the miRs that were elevated in this patient group, namely miR-3148, -30d-5p and 320a. In order to generate an *in vitro* inflammatory model, that would reflect conditions within the SLE patient group, we used the standard model that has been described previously in this group where SMCs were treated with the inflammatory cytokine TNF- α (Parker et al., 2014; McCarthy et al, 2017). The level of miR-3148 was determined in cells and microvesicles isolated from the media aspirated from control (exosome-free DMEM only) and TNF-α-treated cells, by qRT-PCR. This treatment reflects the elevated inflammatory conditions experienced in the vasculature of SLE patients

and allows investigation of the level of miR-3148 in the cells and microvesicles in the extracellular matrix *in vitro*. There was a higher level of miR-3148 expression in cells and microvesicles from the media of cells treated with TNF-α compared to control (Figure 11).

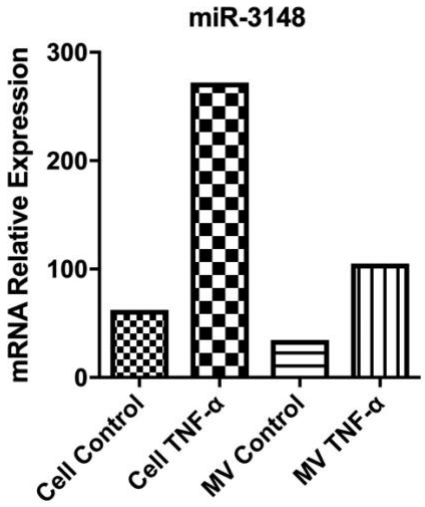

Figure 11 *miR-3148 mRNA expression under inflammatory conditions*

CASMCs were serum starved for 24 hours before treating with TNF-α in exosome-free DMEM for 24 hours. Microvesicles were isolated by ultracentrifugation and miRNA extracted from cells and microvesicles as previously described by our group (McCarthy et al., 2016; Mahmoud et al., 2017). qRT-PCR was carried out to observe the expression of mRNA for miR-3148 where the control was treated with exosome-free DMEM only. The level of miR-3148 expression was higher in cells and microvesicles from media treated with TNF-α compared to control. The data are expressed as densitometric ratios of miR-3148/let-7a-5p. (*n = 1*)

3.2 High Titre Lentiviral Vectors show a stable expression

pLL3.7, a third-generation lentiviral vector, contains the GFP reporter gene and its expression can be visualised under fluorescence microscopy where the cells that have been transfected express the GFP reporter gene. Weekly examination of GFP expression revealed a stable expression over the calcification assay period of 15 - 18 days, as shown in Figure 12. A one-way ANOVA test showed an increase in the percentage of transfected cells (68.8 ± 1.47% at 72 hours, 88.1 ± 0.90% at day 7 - 9 and 97.0 ± 0.5% at day 15 - 17, *P = 0.0001*).

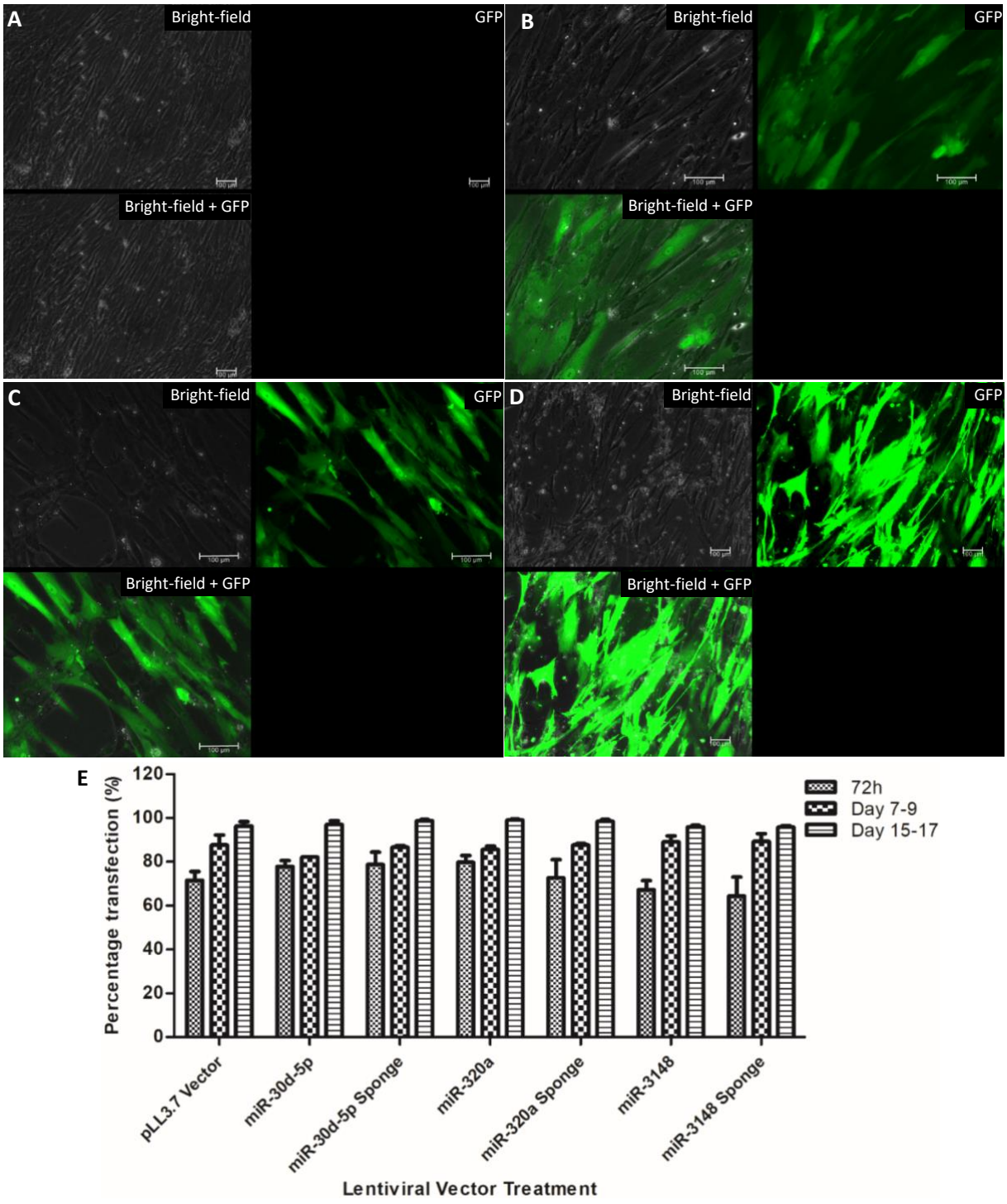

Figure 12 *Third-generation lentivirus efficiently transduce CASMCs*

GFP expression (green) visualised under a live-cell imaging fluorescent microscope at 72 hours without lentiviral vector **(A)** and 72 hours (68.8 ± 1.47%) **(B)**, day 7 - 9 (88.1 ± 0.90%) **(C)** and day 15 - 17 (97.0 ± 0.5%) **(D)** after transfecting CASMCs with miRNA-3148 lentivirus. Transfection of cells was quantified by counting cells infected with the lentivirus (green) and total number of cells in the image, for each of the miRNA and their respective sponges, and pLL3.7 vector **(E)**. A two-way analysis of variance (2-way ANOVA) shows an increase in the percentage of transfected cells with time (*P = 0.0001*). Data are representative images of each group of cells and data is expressed as mean ± S.E.M. (*n = 4*)

3.3 Effect of miRNA-30d-5p and -320a on calcification of coronary artery smooth muscle cells

Cells were transfected with miR-30d-5p and 320a (Figure 13A & C) and their respective sponge controls (Figure 13B & D) respectively. An empty control viral vector pLL3.7 was used as transfection control (Figure 13E). All cells were cultured in osteogenic media containing high calcium (2.6 mmol/L) and βGP (5 mmol/L). The empty vector control cells deposited a mineralised matrix at day 18, which stained positive (red) with ARS and being the highest level of mineralisation taken as 100% (Figure 13E). Cells transfected with miR-30d-5p and -320a appeared to decrease mineralisation *vs* empty vector control (Figure 13A, C & F) (68.6 ± 7.15% and 52.4 ± 6.5% respectively). However, the respective sponge controls (Figure 13B, D & F) also produced less mineralisation compared to the empty control vector (61.6 ± 7.5% and 47.2 ± 6.5% respectively), but no significant difference to their respective miR mimicas (Figure 13F). The levels of Alizarin Red staining was quantified and summarised in Figure 13F.

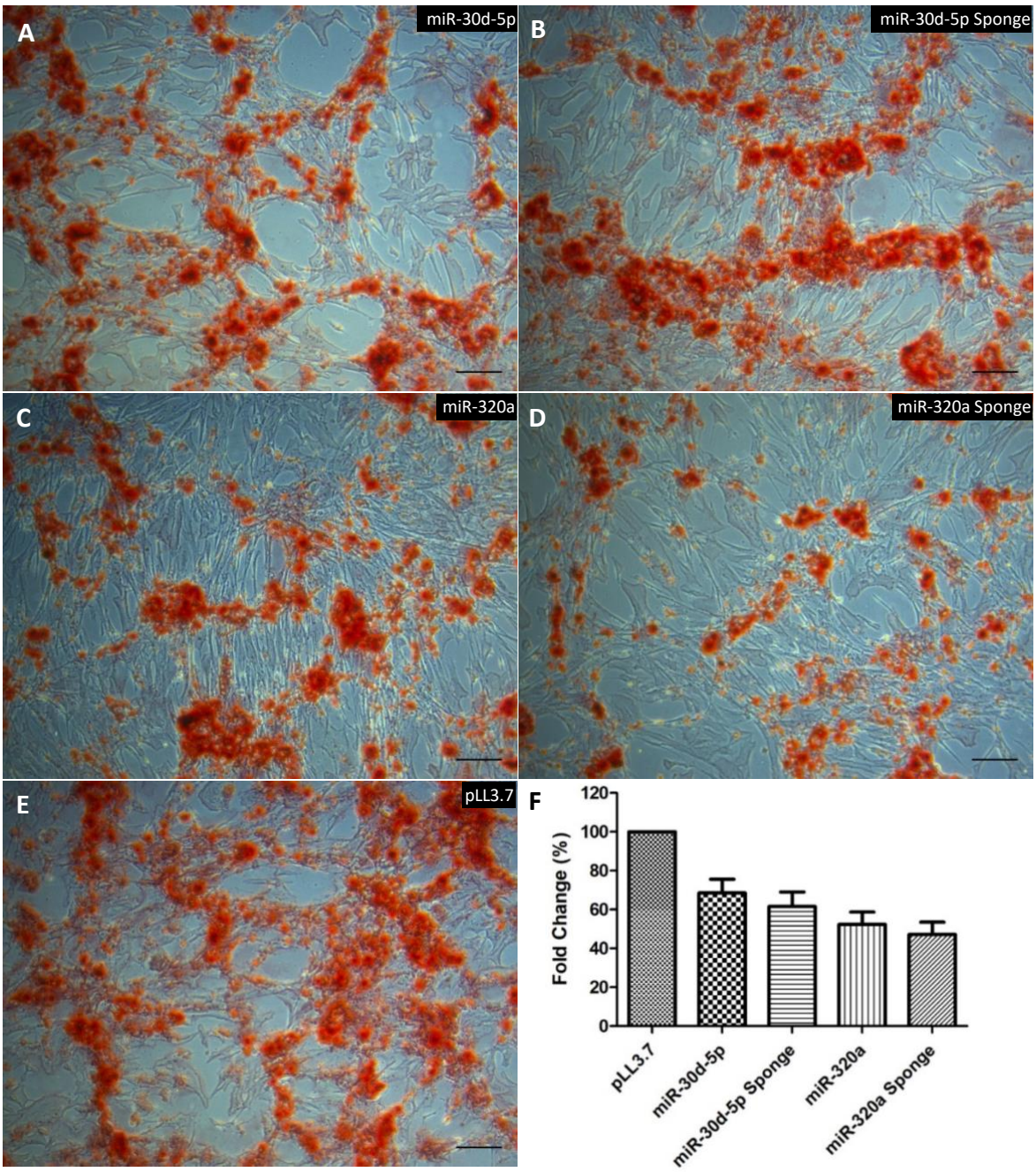

Figure 13 *ARS staining of CASMC growth in osteogenic media*

Mineralisation from the empty vector was taken as 100% and the test miRNAs were expression as a % relative to this. CASMCs transduced with miR-30d-5p **(A)**; miR-30d-5p sponge **(B)**; miR-320a **(C)**; miR-320a sponge **(D)**; and control empty pLL3.7 vector **(E)** were stained at day 15 – 18 of treatment. ARS staining of calcified CASMCs was quantified via elution with 10% Formic acid and measured spectrophotometrically at 414 nm **(F)**. Data are representative images of each group of cells and data is expressed as mean ± S.E.M. (*n = 2*), and the value of the control cells were designated as 100%.

3.4 Overexpression of miRNA-3148 affects calcification *in vitro*

Confluent CASMCs were transfected with either empty pLL3.7 vector, miR-3148 lentivirus or miR-3148 sponge vector and cultured in osteogenic media to induce mineralisation. A one-way ANOVA test indicated an apparent lower calcific matrix formation in cells transfected with miR-3148 compared to control, empty pLL3.7 lentiviral vector, by quantification of ARS staining (73.4 ± 8.1% and 100%, respectively, $P = 0.0201$) (Figure 14A, C & D). Fold change amounted to 79.5 ± 11.4% for miR-3148 sponge (Figure 14 B & D).

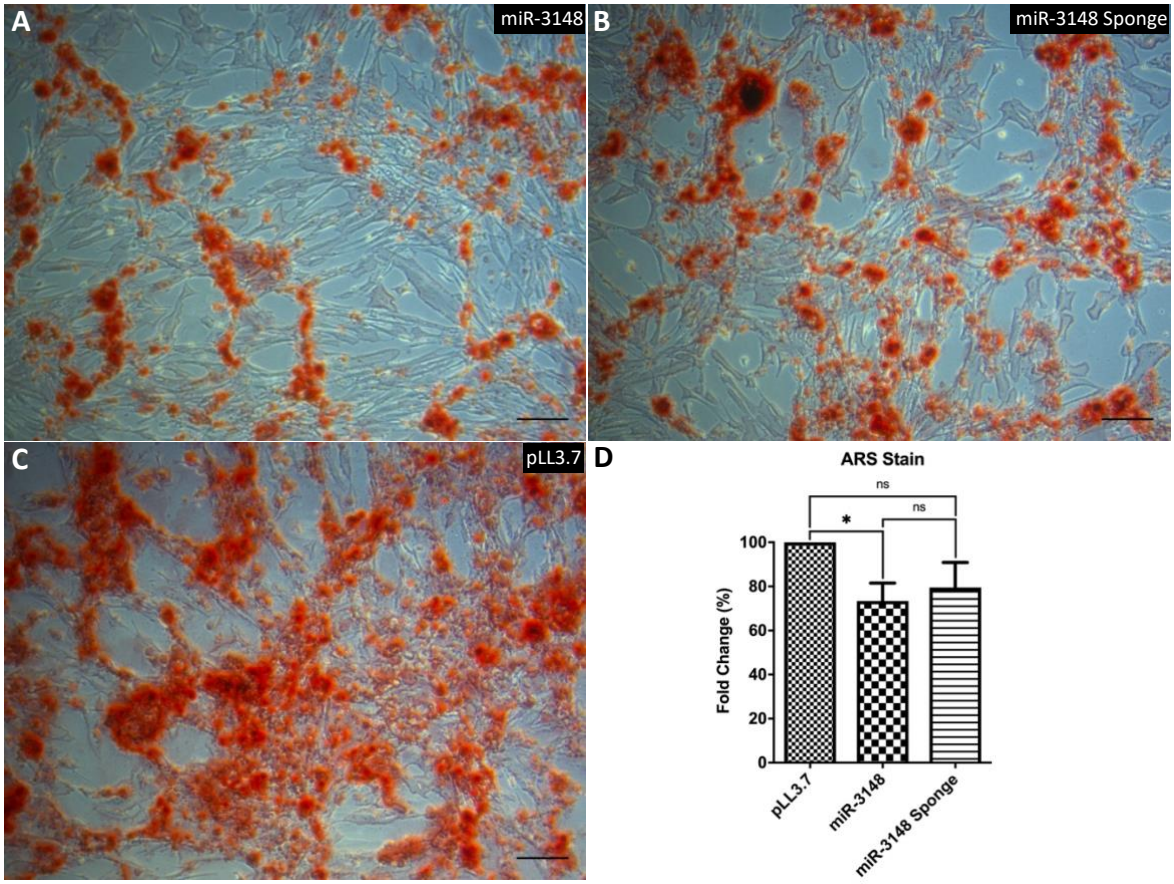

Figure 14 *miR-3148 reduces calcific matrix formation*

CASMCs transduced with miR-3148 **(A)**; miR-3148 Sponge **(B)**; and control empty pLL3.7 vector (100%) **(C)** was stained with ARS at day 18 of treatment. ARS staining of calcified CASMCs were quantified via elution with 10% Formic acid and measured spectrophotometrically at 414nm **(D)**. Data are representative images of each group of cells and data is expressed as mean ± S.E.M. (*n = 4*), and the value of the control cells were designated at 100%. * = significant difference (*P = 0.0201*), ns = no significant difference

3.5 miR-3148 Reduces Runx2 Gene Expression

In order to identify the pathways through which miR-3148 was executing its effects, the next series of experiments was carried out to determine whether this transcription factor was in fact targeted by miR-3148. Among the many Runx2-targeting miRNAs, miR-3148 has been suggested as a potential modulator of VC via regulation of Runx2 expression. At day 4, CASMCs in osteogenic media transfected with miR-3148 showed lower expression of Runx2 compared to the control (0.061 ± 0.030 fold change vs 0.238 ± 0.157 fold change) and compared to miR-3148 sponge (0.171 ± 0.122 fold change). Although there was a trend towards reduction in Runx2 mRNA abundance in cells transfected with miR-3148 viral vector, the difference was not significant. Runx2 expression was lower in cells transfected with the miR-3148 sponge compared to control (Figure 15).

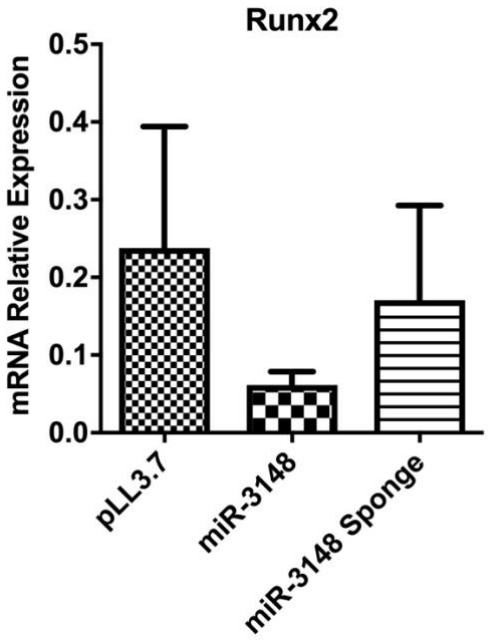

Figure 15 *miR-3148 negatively regulates calcific matrix formation*

qRT-PCR was conducted on RNA extracted from CASMCs at day 4 of the calcification assay. Expression of Runx2 mRNA in pLL3.7, miR-3148 and miR-3148 sponge lentivirus transfected cells, relative to GAPDH expression, is shown. Data are expressed as mean ± S.E.M. (*n = 4*), and the values of the control gene, GAPDH, expression were designated as 1. Significant difference was not observed between the different treatments.

Western blot analysis was conducted to investigate the expression of the Runx2 protein at day 4 in cells cultured in osteogenic media. It is difficult to draw a conclusion from the results for

Runx2 protein stain (Figure 16) as there was no evidence of the presence of Runx2 protein. The loading control, α-tubulin, was present in all protein samples. The reason for the lack of detectable expression is unclear, but maybe a later time-point in the mineralisation process will yield a different result.

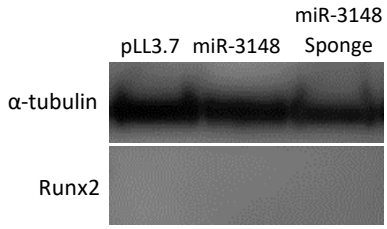

Figure 16 *Protein level at day 4*

Total protein cell extracts (15 µg) of CASMCs were electrophoresed using 4-12% Bis-Tris gel. Western blotting was performed by probing with purified anti-Runx2 (57 kDa), with an anti-α-tubulin (50 kDa) antibody being used as loading control. (*n = 1*)

3.6 Changes in ALP activity

In support of previous findings using ALP activity as an early marker of osteogenic differentiation, a small increase was observed at day 4 in cells from all treatments (pLL3.7 0.000199 ± 0.0.00379 nmol/L; miR-3148 0.00108 ± 0.00391 nmol/L; miR-3148 sponge 0.00086 ± 0.0.00525 nmol/L), with the highest increase in cells transfected with miR-3148 lentivirus. At day 7, a small increase in ALP activity in control cells and a comparatively higher increase in cells with elevated levels of miR-3148 (0.00049 ± 0.00366 nmol/L and 0.00358 ± 0.00394 nmol/L respectively) was observed. There was a drop in ALP activity in cells transfected with miR-3148 sponge (-0.00077 ± 0.00387 nmol/L) as shown in Figure 17.

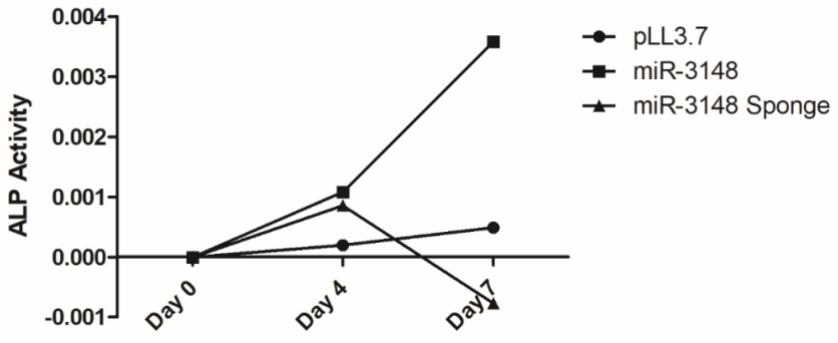

Figure 17 *ALP activity of cells cultured in osteogenic media*

CASMCs were seeded in 6-well plates and transfected with lentiviral vectors in osteogenic media. Protein was isolated and quantified using BCA assay and then ALP activity assay performed. Absorbance of protein solutions from each well was measured at 405nm. (*n = 1*)

3.7 Altered expression of calcification-related genes

Figure 18 displays the level of gene expression of the chosen calcification-related genes under the various conditions. BMP2 is a predicted target for miR-3148, however, the expression of BMP2 is higher in cells transfected with miR-3148 (0.00256 ± 0.00216 fold change) than control (0.00085 ± 0.00066 fold change) and less than in cells transfected with miR-3148 sponge (0.00505 ± 0.00476 fold change). Sirt1 gene expression was lowest in cells transfected with miR-3148 compared to the control and sponge. Sirt1 gene expression analysis amounted to 0.00493 ± 0.00231-fold change, 0.00303 ± 0.00167-fold change and 0.00687 ± 0.00203-fold change for cells transfected with empty pLL3.7, miR-3148 and miR-3148 sponge, respectively. Highest level of Sox9 expression was observed in cells transfected with miR-3148. Sox9 gene expression analysis amounted to 0.00045 ± 0.00027-fold change, 0.001229 ± 0.00059-fold change and 0.00094 ± 0.000281-fold change for control, miR-3148 and miR-3148 sponge transfected cells, respectively. Levels of MMP16 (Matrix metalloproteinases 16) was highest in miR-3148 transfected cells. The MMP16 gene expression levels amounted to 0.00268 ± 0.00138-fold change, 0.00845 ± 0.00466-fold change and 0.00472 ± 0.00359-fold change for cells transfected with control, miR-3148 and miR-3148 sponge vectors, respectively. These data show no clear trend for BMP2 expression, a trend towards reduction of Sirt1 in cells with elevated miR-3148 levels, and a trend towards elevation of Sox9 and MMP16 in these cells, however, no significant changed was detected.

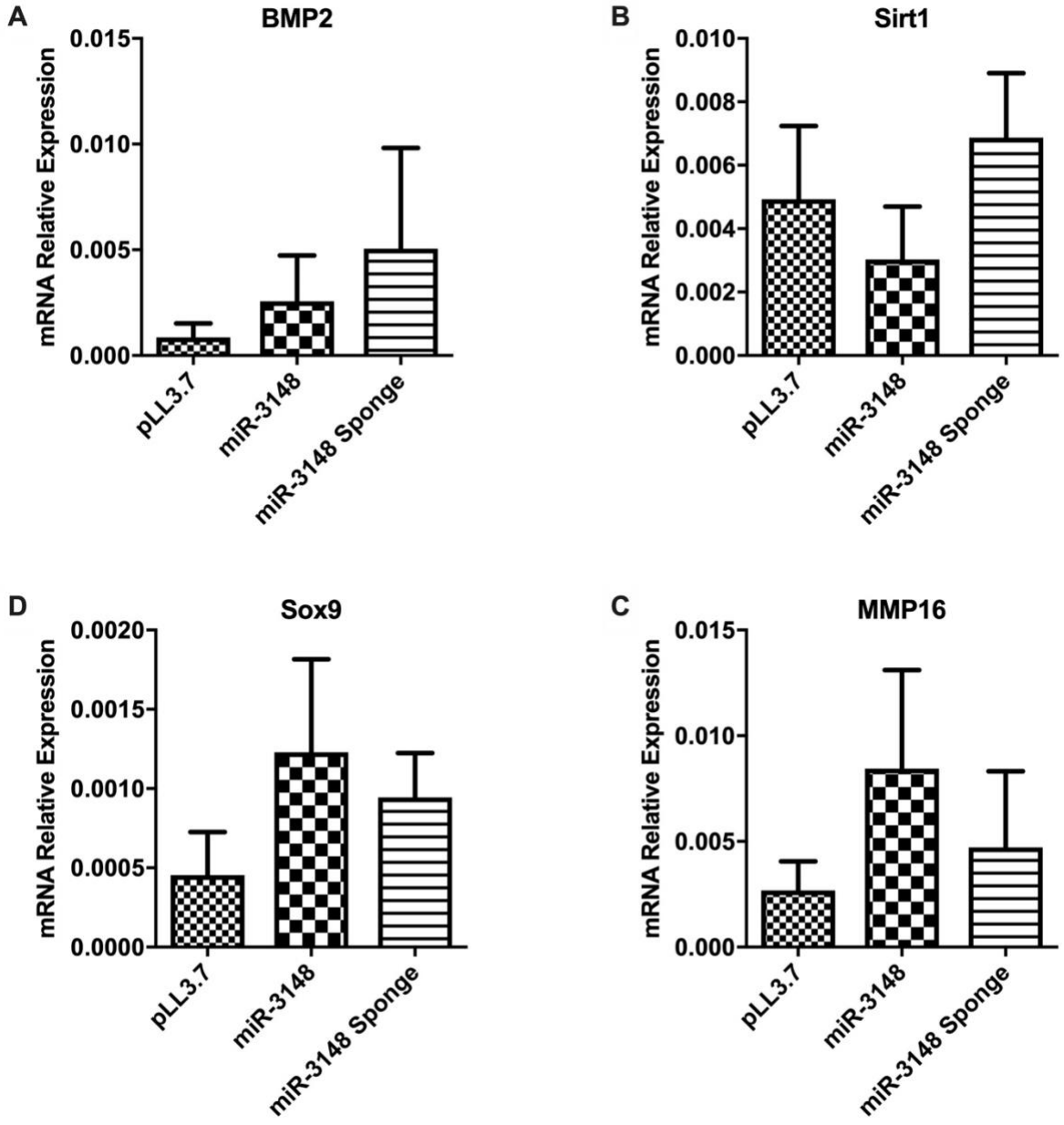

Figure 18 *Gene expression in CASMCs at day 4*

(A-D) CASMCs were transfected with control empty lentiviral vector pLL3.7 or miR-3148 or miR-3148 sponge and subjected to qRT-PCR analysis of BMP2 **(A)**, Sirt1 **(B)**, Sox9 **(C)** and MMP16 **(D)**. Data is expressed as a relative fold change compared with GAPDH mRNA. The data are expressed as mean ± S.E.M. of each group of cells from three separate experiments, and the values of the control gene, GAPDH, expression were designated as 1. Significant difference was not observed between any of the conditions for each of the genes. (*n = 3*)

3.8 Destination Vector for Gateway® Cloning

The DV produced via the BP reaction using Gateway® Cloning technology was extracted from transformed bacteria and analysis of recombinant clones was carried out by investigating the

integrity of the DNA which was established by restriction enzyme digestion and electrophoresis (Figure 19). The presence of a band at 2.7 kb (length of the Gateway® cassette containing *att*R1, *ccd*B, chloramphenicol resistance and *att*R2 genes) confirmed successful selection and amplification of the DV.

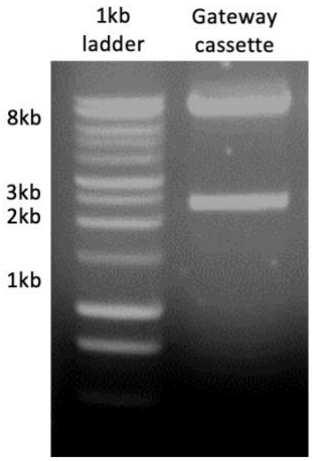

Figure 19 *Electrophoresis of digested destination vector*

Electrophoresis was carried out at 120V for 15 minutes. The lower 2.1kb band in the right column represents the Gateway® cassette sequence containing *attR1*, *ccdB*, CM^R and *attR2* sites.

3.9 Confirmation of Successful Vector Contruction by DNA Sequencing

DNA sequencing was conducted on all vectors containing miRNA and their respective sponges, and the DV produced via BP reaction. Sequencing the vector DNA showed successful amplification of each vector. Figure 20 shows the sequencing results of the Gateway® cassette in the DV.

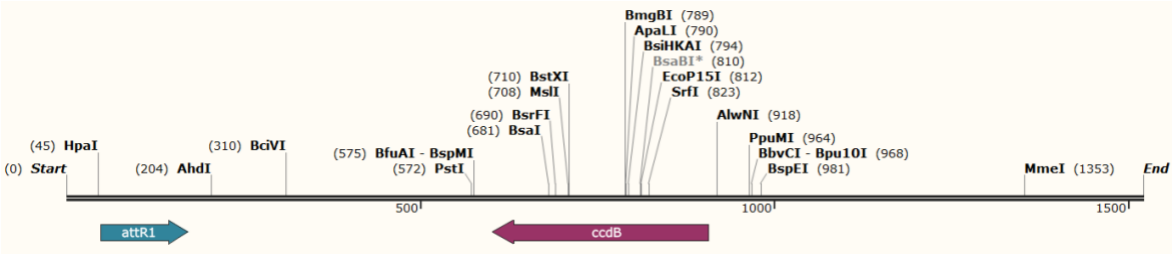

Figure 20 *Sequencing of destination vector*

Sequencing showed the presence of attR1 site in the Destination Vector produced via the BP reaction using enzyme BP Clonase™.

4 Discussion

Recent studies have supported the finding that young women with SLE have a high risk of developing atherosclerosis and subsequent vascular calcification compared to their healthy counterparts, and the severity of the calcification determines cardiovascular mortality and morbidity (Manzi et al., 1997). The aetiology of accelerated coronary artery calcification in these patients is multifactorial, but the precise mechanism underlying the osteogenic differentiation of CASMCs in this patient group remains unclear. Studies from our group have previously shown an increase in the levels of endothelial cell-derived microvesicles in SLE patients and changes in levels of a range of miRNAs compared to control patients (Edwards et al., 2018). Numerous studies have shown a crucial role of miRNAs in modulating CVD. For example, increased miR-133a, -125b and -204 activity inhibit osteoblastic differentiation of VSMCs by targeting Runx2 expression, whereas suppression of activity of these miRNAs induce VSMC osteoblastic differentiation (Goettsch et al., 2011; Cui et al., 2012; Liao et al., 2013). Wang and colleagues (2015) have confirmed the inhibitory effects of miR-204 on the transdifferentiation of human aortic vascular interstitial cells into osteoblast-like cells by targeting Runx2.

TNF-α is a potent inflammatory factor and an immunoregulatory cytokine involved in the pathological basis of endothelial injury and atherosclerosis (López-Pedrera et al., 2010; Zhang et al., 2014). Under inflammatory conditions in the vasculature, TNF-α is primarily released by monocytes and macrophages (Tintut et al., 2000; Durham et al., 2018). In cultured human CASMCs, the addition of TNF-α stimulated production of miR-3148 within the cells and in microvesicles released from the cells. These results correlate with the increase in miR-3148 in the peripheral blood of patients with SLE as shown by preliminary data generated by our group. Complementary studies, carried out in our group by Dr Alex Langford-Smith and Nicola Edwards, show significantly elevated levels of miR-3148 in microvesicles isolated from SLE patients (*n = 26)* compared to controls (*n = 14*), *P = 0.001* (unpublished data). Furthermore, many studies have revealed an increase in release of EMVs from activated endothelial cells and cultured human umbilical vein endothelial cells in response to TNF-α (Buendía et al., 2015; McCarthy et al., 2016), suggesting the source of EMVs and miR-3148 may be endothelial cells in addition to CASMCs. Work by Tintut et al. (2000) demonstrated an upregulation of Runx2

expression in response to stimulation by TNF-α in *in vitro* studies on calcification of bovine VSMC cultures.

In the present study, to investigate the role of miR-30d-5p, -320a and -3148, human CASMCs were transduced with third-generation lentivirus containing synthetic miRNA mimics or sponges for altered miRNA activity, in an *in vitro* model of calcification. To decrease the level of miRNA, miRNA sponges were produced and introduced to the cells using lentivirus. Lentiviral vectors were producing using three vectors; (a) a third-generation packaging plasmid pCMV-dR8.91 coding for vital components of the virion, derived from HIV-1; (b) an enveloped protein plasmid that codes for VSV-G, due to its broad tropism and high stability (Klages et al., 2000) and (c) pLL3.7 plasmid encoding the target gene. The third-generation packaging plasmid used contains three genes of HIV-1: *gag*, codes for the core structural proteins of the virion; *pol*, encodes retrovirus-specific enzymes; and *rev*, codes for a post-transcriptional regulator required for *gag* and *pol* expression (Klages et al., 2000).

The changes caused by transfecting cells to alter miRNA levels were observed in an *in vitro* calcification assay model using ARS staining and quantification methods, followed by determining the expression of genes associated with calcification. The process of calcification under pathological conditions occurs over decades, so an *in vitro* model to mimic this process was generated using high glucose DMEM supplemented with 2.6 mmol/L $CaCl_2$ and 5 mmol/L βGP, for 15 to 18 days, depending on the experiment. This is the first study to assess the role of miRs in the pathology of calcification, which many be associated with the pathology of SLE. Although *n* numbers were small and did not allow statistical analysis to be calculated, the data generated suggest that the miR-3148 inhibits CAC in CASMCs cultured in osteogenic media *in vitro*. There was a lower level of mineralisation in cells transfected with miR-3148 lentivirus compared to control (empty pLL3.7 vector). This correlates with the trend towards a decrease in expression of Runx2 gene in these cells at day 4, under the same condition. Runx2 is a transcription factor which encodes a nuclear protein which is essential for osteogenic differentiation of cells and skeletal morphogenesis via multiple pathways. In VSMCS, Runx2 is able to regulate expression of VSMC marker genes under oxidative stress, including myocardin and serum response factor (SRF), a transcription factor crucial in modulating cell growth and differentiation (Sun et al., 2012; C. Chen et al., 2015; Frismantiene et al., 2018). The

upregulation of Runx2 may disturb the formation of SRF/myocardin complexes and participate in the decreased expression of SMC gene markers (Sun et al., 2012). Consequently, Runx2-deficient VSMCs have normal expression of SMC markers, allowing the cells to perform their normal functions under healthy physiological conditions. In addition, under normal conditions the vasculature expresses mineralisation inhibitors such as feutin-A and matrix Gla protein (MGP) which abrogates mineralisation (Reynolds et al., 2004). Expression of bone mineralisation and matrix proteins are also regulated by Runx2 (Zamurovic et al., 2004). The phenotypic changes in the cells under disease conditions allow them to express calcification-regulating proteins that are normally confined to bone and cartilage (Shanahan et al., 1999). Moreover, Runx2 is known to affect the expression of different miRNAs, for example, it is able to bind to the promoter regions of miR-23a, -27a and -24-2 cluster involved in mineralisation, limiting their expression (Hassan et al., 2010). This illustrates the complex system of miRNA gene regulation.

miRNA targets and target prediction scores were identified using DIANA-TarBase algorithms (Vlachos et al., 2015). miR-3148 targets Runx2 (target prediction score: 0.9988), and therefore, downregulates Runx2 expression, leading to reduced secretion of osteocalcin and osteopontin among other factors supporting calcification (Zamurovic et al., 2004), followed by a reduced CASMC differentiation into osteogenic-like cells and consequential decrease in mineralisation. These data support the findings of Sun et al. (2012) where evidence was reported of osteogenic differentiation of VSMCs contributing to vascular calcification, and the importance of Runx2 expression within VSMCs in regulating the calcification process. During development, Runx2 expression precedes differentiation of cells by several days, hence why gene expression was analysed at day 4. Changes in Runx2 protein expression under the different lentiviral vectors was also examined, but Runx2 proteins were not observed in the Western blot analysis which may be due to the anti-Runx2 antibody which was used, or the possibility that the protein was in too low abundance to be detected. In the interest of time, Western blot optimisation was not carried out in this case. The presence of bands in the control stain for protein α-tubulin confirms good transfer/blotting and suggests issues with the Runx2 primary antibody used. The possible problems may have been that the concentration of primary antibody used was too low or a cross-reactivity between the blocking agent and the primary antibody may have occurred.

Furthermore, it has been acknowledged that Runx2 triggers ALP activity (Lian et al., 2004), an early marker of osteogenic differentiation (Liu et al., 2011), so ALP activity was expected to be lower in cells transfected with miR-3148 lentivirus compared to the control and sponge. ALP degrades inorganic pyrophosphates, a major inhibitor of hydroxyapatite crystal growth, and releases phosphate (Kapustin et al., 2011) and it is generally a classic marker of osteogenic differentiation. Results from the ALP activity assay were inconclusive due to negative values and the presence of outliers. This may be the result of technical error with pipetting, a very low concentration of protein used, or an incorrect incubation period.

Several calcification-related genes were found to be targets for the differentially expressed miRNAs at day 4, when a screen was performed using the software analysis programme StepOne Plus. Expression of the calcification genes BMP2, Sirt1, Sox9 and MMP16 in CASMCs transfected with control, miR-3148 and miR-3148 sponge lentivirus was investigated using total extracted RNA. BMP2 expression was expected to be lower under high miR-3148 activity compared to control and to correlate with the results for Runx2 expression as it induces the expression of Runx2 (Zhao et al., 2016), but the opposite was seen. BMP2 expression was lowest in control and highest in miR-3148 sponge lentivirus-transfected cells. As aforementioned, many miRNAs have the same target (e.g. Runx2 is downregulated by miR-30b-d, but upregulated by miR-32) (Nanoudis et al., 2017) while a single miRNA may target more than one gene and affect different pathways with opposing results. Moreover, the target prediction of miR-3148 for BMP2 is very low (target prediction score: 0.7095) compared to Runx2, which means Runx2 expression was highly targeted compared to BMP2, explaining the difference in expression between the two genes.

miR-3148 targets regulation of Sirt1 (target prediction score: 0.9855), a gene related to cell longevity and a marker for cell senescence (Takemura et al., 2011). Sirt1 downregulation has been observed in smooth muscle cells during osteoblastic differentiation (Takemura et al., 2011). Recent studies have portrayed the correlation between increasing senescence-associated secretory phenotype and increase in the tendency of VSMCs to undergo osteogenic differentiation (Takemura et al., 2011; Badi et al., 2018). In addition, senescent VSMCs are characterised by the expression of Runx2, among other bone-regulated genes (Badi et al., 2018). A study conducted by Takemura et al. (2011) demonstrated Sirt1 to be an inhibitor of

vascular calcification by impeding VSMC senescence. Interestingly, this is contradicted by gene expression data from the present study which shows a reduced level of calcification in cells with downregulation of Sirt1 as a result of high miR-3148 activity, compared to control and miR-3148 sponge. This suggests that the miR-3148-mediated downregulation of Sirt1 does not have an effect on mineralisation and differentiation of CASMCs. This may be due to other aspects in this multifactorial disease.

Sox9, a chondrogenic regulator primarily expressed in resting and proliferating chondrocytes, is also targeted by miR-3148 (target prediction score: 0.9905) (Zhang et al., 2012; Loebel et al., 2015). It directly interacts with and represses the activity of Runx2. This is supported by the negative correlation seen between Sox9 and Runx2 gene expression and mineralisation levels under the different treatments of CASMCs in this study. The Runx2 and Sox9 gene expression under osteogenic conditions may reflect the interaction between the two transcription factors. Accordingly, an increase in Runx2/Sox9 ratio correlates with an increase in calcification and vice versa. Likewise, a lower Sox9 expression compared to Runx2 was detected in osteo-chondroprogenitor cells differentiating towards osteogenic phenotypes during endochondral bone formation in mice (Akiyama et al., 2005). Sox9 binds to a target sequence on Runx2 and inhibits its function by either preventing protein degradation of the Runx2 gene or the activation of osteoblast-specific enhancers (Zhou et al., 2006; Cheng and Genever, 2010; Loebel et al., 2015). However, the correlation between Sox9 gene expression and Runx2 protein could not be established due to poor Western blot results. Lowest expression of Sox9 was expected in cells transfected with miR-3148 lentivirus, but the opposite was seen, this may be due to the low target prediction score for Sox9, as well as involvement of other factors regulating translation of the Sox9 gene. Future studies may involve elucidating the knockdown effects of Sox9 on various inhibitory genes to better the understanding of this regulatory network (Loebel et al., 2015).

MMP16 gene translation is regulated by miR-3148 (target prediction score: 0.9995), so a reduced MMP16 expression was expected in cells in comparison to control and miR-3148 sponge. However, this was not supported by the data produced in the present study. The highest MMP16 expression was observed in cells transfected with miR-3148 and the lowest in control. This suggests that changes in MMP16 may be due to Runx2-driven vascular

calcification. There is a positive correlation between expression of MMP16 and changes in calcification. Currently, no data is available on the association between MMP16 and Runx-2-driven vascular calcification. More investigation is required to elucidate the mechanism of this enzyme in the current disease pathology.

A reduction in calcification was also observed in cells transfected with lentivirus for miR-30d-5p and -320a, but not in cells transfected with the sponge for these miRNAs, so this study focused on miR-3148. These results may be explained by the fact that miRNA sponges are able to inhibit all seed family members (Barta et al., 2016). In addition, miRNA sponges may display off-target effects by binding to unspecific miRNAs (Barta et al., 2016). On the other hand, each of the miRNAs are predicted to have hundreds of other targets, and are targeted by other components of the pathway, thus, regulation of calcification-related gene expression may involve a large network associated with other biological processes that work to maintain vascular homeostasis.

5 Limitations of the study

Several limitations are applicable to this study. Firstly, time constraints during this project did not allow for optimisation and completion of certain experiments including Western blot analysis and the ALP activity assay. It is essential to observe the effects of altered miR-3148 activity on Runx2 protein level to confirm direct interaction with Runx2 protein. Previous research involving miR-204 has shown changes in Runx2 protein, but no change in Runx2 mRNA translation in mouse and human mesenchymal stem cells (Huang et al., 2010). Moreover, in the current study, although it is shown that miR-3148 successfully reduces calcification, it does not show how the miRNA is modulated during the natural process of vascular calcification.

In addition, primary cells were used for the experimental procedures. In an *in vitro* culture, primary human CASMCs experience dedifferentiation in response to high concentration of growth factors in the culture media (Worth et al., 2001). However, primary human cells have limitations which can lead to irreproducibility or formation of artefacts due to the fact that the cells have been removed from their microenvironment (Frismantiene et al., 2018). Furthermore, cells from different vascular beds have different properties and those from large

vessels like aortic smooth muscle cells have a greater plasticity than those from micro-vessels such as the CASMCs, where each can respond differently to the stimuli due to the nature of the heterogenic characteristics (Majesky, 2007). High heterogeneity can also be present in the same segment of the vessel, and therefore, inaccuracies in isolation of the tissue can cause high variability in primary culture (Frismantiene et al., 2018), making the experiment irreproducible. This variation in methods of CASMC isolation and cultivation can explain conflicting results between different laboratories (Alexander and Owens, 2012). Additionally, human CASMC isolates have a limited lifespan and are sensitive to handling and culture, in contrast to transformed cell lines. These isolates are prone to immediate senescence and are affected by donors' age, gender and the applied treatments (Frismantiene et al., 2018).

Moreover, *in vitro* studies eliminate environmental cues such as three-dimensionality and blood flow stimulation. In blood vessel walls VSMCs are elongated and arranged in an organised manner in a circumferential direction, whereas within *in vitro* cultures they are less elongated and randomly distributed on the culture plates (Chang et al., 2014). In addition, CASMCs could not be used beyond passage 8 due to their high tendency to undergo differentiation and change in phenotype. Research has shown that VSMCs undergo passage-associated spontaneous phenotypic switching within *in vitro* culture studies, making it difficult to identify predictive markers of pathologic state (Chang et al., 2014). Despite these limitations, this study shows strong evidence of the effects of changes in miR-3148 activity on Runx2-regulated vascular calcification via osteogenic differentiation of CASMCs.

6 Future Directions

The current data shows long-term transfection of lentivirus in cultured CASMCs by assessment of GFP expression, suggesting stable increase in miRNA and miRNA sponge activity over the course of the calcification assay due to the ability of lentiviral vectors to integrate in the host cell genome (Segura et al., 2007). However, the level of miRNA in transfected cells was not determined, and this would need to be confirmed in future studies. Moreover, it has been suggested that certain miRNAs may have a tissue-specific nature of expression. An example of this is the opposite effects of miR-221-3p found in endothelial cells and VSMCs (Liu et al., 2012). Another factor to consider is that certain miRNAs can behave differently in combination

with another. For example, a mouse study showed an increase in calcium deposition when miR-221 and -222 were combined, but not when applied separately (Mackenzie et al., 2014).

Studies in the future could investigate the effects of altered miR-3148 activity in different cell types and in conjunction with other miRNAs within the vasculature on calcification-related gene expression. Moreover, a longitudinal study on the effect of changes in miR-3148 and onset of vascular calcification in SLE patients would be beneficial in helping to elucidate the mechanisms of miR-3148 in this pathology.

Gateway® Cloning technology was used to produce destination vectors via the BP reaction. The production of this vector will allow the rapid production of miRNA and sponge expression constructs in the future. This cloning system is advantageous due to its rapid, high-throughput generation of Expression Clones and availability of a wide choice of open reading frames (Doyle, 2009).

7 Conclusion

This is the first study to investigate the regulatory role of EMV-derived miR-3148 in CAC via targeting Runx2 gene expression. Collectively, this report supports the hypothesis and suggests a protective role for miR-3148 against osteogenic differentiation of CASMCs and subsequent CAC in SLE patients. MV-associated miR-3148 is overexpressed in patients with SLE and it reduces osteogenic transdifferentiation of CASMCs *in vitro* when stimulated with βGP. This suggests that miR-3148 has a protective mechanism. However, a reduction in CAC does not necessarily translate into better clinical outcomes and cardiovascular risk is still high among this patient population, also signifying that the high level of miR-3148 is insufficient to be protective in this setting and a greater activity could be beneficial. The emerging role of EMVs and miRNAs is promising in reducing overall CVD mortality and morbidity, but further investigation is required to elucidate the precise effects of EMV-miRNA treatment of CAC. This will lead to a better understanding of the *in vivo* mechanisms of EMVs and miRNAs and their implications in drug designs against specific gene targets for the prevention or treatment of CAC.

REFERENCES

Aikawa, E., Nahrendorf, M., Figueiredo, J. L., Swirski, F. K., Shtatland, T., Kohler, R. H., Jaffer, F. A., Aikawa, M. and Weissleder, R. (2007) 'Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo.' *Circulation*, 116(24) pp. 2841-2850.

Akiyama, H., Kim, J. E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., Zhang, Z., Martin, J. F., Behringer, R. R., Nakamura, T. and de Crombrugghe, B. (2005) 'Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors.' *Proceedings of the National Academy of Sciences of the United States of America*, 102(41) pp. 14665-14670.

Alexander, M. R. and Owens, G. K. (2012) 'Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease.' *Annual Review of Physiology*, 74, March, pp. 13-40.

Anglicheau, D., Muthukumar, T. and Suthanthiran, M. (2010) 'MicroRNAs: small RNAs with big effects.' *Transplantation*, 90(2) pp. 105-112.

Badi, I., Mancinelli, L., Polizzotto, A., Ferri, D., Zeni, F., Burba, I., Milano, G., Brambilla, F., Saccu, C., Bianchi, M. E., Pompilio, G., Capogrossi, M. C. and Raucci, A. (2018) 'miR-34a promotes vascular smooth muscle cell calcification by downregulating SIRT1 (sirtuin 1) and Axl (AXL receptor tyrosine kinase).' *Arteriosclerosis, Thrombosis and Vascular Biology*, 38(9) pp. 2079-2090.

Barta, T., Peskova, L. and Hampl, A. (2016) 'miRNAsong: a web-based tool for generation and testing of miRNA sponge constructs in silico.' *Scientific Reports*, 6:36625, pp. 1-8. [Online] [Accessed on 14th January 2018] DOI: 10.1038/srep36625

Bartoli-Leonard, F., Wilkinson, F.L., Schiro, A., Inglott, F.S., Alexander, M.Y., Weston, R. (2019) 'Suppression of SIRT1 in Diabetic Conditions Induces Osteogenic Differentiation of Human Vascular Smooth Muscle Cells via RUNX2 Signalling.' *Scientific Reports*, 9:878, pp. 1-16. [Online] [Accessed on 7th May 2019] DOI: 10.1038/s41598-018-37027-2

Bernard, P. and Couturier, M. (1992) 'Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes.' *Journal of Molecular Biology*, 226(3) pp. 735-745.

Boström, K., Watson, K. E., Horn, S., Wortham, C., Herman, I. M. and Demer, L. L. (1993) 'Bone morphogenetic protein expression in human atherosclerotic lesions.' *Journal of Clinical Investigation*, 91(4) pp. 1800-1809.

Boström, K. I. (2016) 'Where do we stand on vascular calcification?' *Vascular Pharmacology*, 84, September, pp. 8-14.

Brodsky, S. V., Zhang, F., Nasjletti, A. and Goligorsky, M. S. (2004) 'Endothelium-derived microparticles impair endothelial function in vitro.' *American Journal of Physiology-Heart and Circulatory Physiology*, 286(5) pp. H1910-1915. [Online] [Accessed on 25th March 2018] DOI: 10.1152/ajpheart.01172.2003

Buendía, P., Montes de Oca, A., Madueño, J. A., Merino, A., Martín-Malo, A., Aljama, P., Ramírez, R., Rodríguez, M. and Carracedo, J. (2015) 'Endothelial microparticles mediate inflammation-induced vascular calcification.' *FASEB Journal*, 29(1) pp. 173-181.

Burger, D., Schock, S., Thompson, C. S., Montezano, A. C., Hakim, A. M. and Touyz, R. M. (2013) 'Microparticles: biomarkers and beyond.' *Clinical Science*, 124(7) pp. 423-441.

Chang, S., Song, S., Lee, J., Yoon, J., Park, J., Choi, S., Park, J. K., Choi, K. and Choi, C. (2014) 'Phenotypic modulation of primary vascular smooth muscle cells by short-term culture on micropatterned substrate.' *PLOS ONE*, 9(2), pp. 1-10. [Online] [Accessed on 10th July 2018] DOI: 10.1371/journal.pone.0088089

Chang, S. M., Nabi, F., Xu, J., Peterson, L. E., Achari, A., Pratt, C. M. and Mahmarian, J. J. (2009) 'The coronary artery calcium score and stress myocardial perfusion imaging provide independent and complementary prediction of cardiac risk.' *Journal of the American College of Cardiology*, 54(20), pp. 1872-1882.

Chen, C., Wang, Y., Yang, S., Li, H., Zhao, G., Wang, F., Yang, L. and Wang, D. W. (2015) 'MiR-320a contributes to atherogenesis by augmenting multiple risk factors and down-regulating SRF.' *Journal of Cellular and Molecular Medicine*, 19(5) pp. 970-985.

Chen, W., Yu, F., Di, M., Li, M., Chen, Y., Zhang, Y., Liu, X., Huang, X. and Zhang, M. (2018) 'MicroRNA-124-3p inhibits collagen synthesis in atherosclerotic plaques by targeting prolyl 4-hydroxylase subunit alpha-1 (P4HA1) in vascular smooth muscle cells.' *Atherosclerosis*, 277, August, pp. 98-107.

Cheng, A. and Genever, P. G. (2010) 'SOX9 determines RUNX2 transactivity by directing intracellular degradation.' *Journal of Bone and Mineral Research*, 25(12), pp. 2680-2689.

Condorelli, G., Latronico, M. V. and Cavarretta, E. (2014) 'microRNAs in cardiovascular diseases: current knowledge and the road ahead.' *Journal of the American College of Cardiology*, 63(21) pp. 2177-2187.

Cui, R. R., Li, S. J., Liu, L. J., Yi, L., Liang, Q. H., Zhu, X., Liu, G. Y., Liu, Y., Wu, S. S., Liao, X. B., Yuan, L. Q., Mao, D. A. and Liao, E. Y. (2012) 'MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo.' *Cardiovascular Research*, 96(2) pp. 320-329.

Dangwal, S. and Thum, T. (2014) 'microRNA therapeutics in cardiovascular disease models.' *Annu Rev Pharmacol Toxicol*, 54, January, pp. 185-203.

Demeke, T., Ratnayaka, I. and Phan, A. (2009) 'Effects of DNA extraction and purification methods on real-time quantitative PCR analysis of Roundup Ready soybean.' *Journal of AOAC International*, 92(4) pp. 1136-1144.

Demer, L. L. and Tintut, Y. (2003) 'Mineral exploration: search for the mechanism of vascular calcification and beyond: the 2003 Jeffrey M. Hoeg Award lecture.' *Arteriosclerosis, Thrombosis and Vascular Biology*, 23(10) pp. 1739-1743.

Demer, L. L. and Tintut, Y. (2008) 'Vascular calcification: pathobiology of a multifaceted disease.' *Circulation*, 117(22) pp. 2938-2948.

Distler, J. H., Pisetsky, D. S., Huber, L. C., Kalden, J. R., Gay, S. and Distler, O. (2005) 'Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases.' *Arthritis & Rheumatology*, 52(11) pp. 3337-3348.

Doyle, S. (2009) *High throughput protein expression and purification*. Walnut Creek, California: Humana Press, pp.31-34.

Durham, A. L., Speer, M. Y., Scatena, M., Giachelli, C. M. and Shanahan, C. M. (2018) 'Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness.' *Cardiovascular Research*, 114(4) pp. 590-600.

Edwards, N., Langford-Smith, A., Parker, B., Bruce, I., Reynolds, J., Alexander, Y., McCarthy, E., and Wilkinson, F. (2018) 'QRISK3 improves detection of cardiovascular disease risk in patients with Systemic Lupus Erythematosus.' *Lupus Science & Medicine*, 5(1), pp. 1-6 [Online] [Accessed 7th May 2019] DOI: 10.1136/lupus-2018-000272

El-Magadmi, M., Bodill, H., Ahmad, Y., Durrington, P. N., Mackness, M., Walker, M., Bernstein, R. M. and Bruce, I. N. (2004) 'Systemic lupus erythematosus: an independent risk factor for endothelial dysfunction in women.' *Circulation*, 110(4) pp. 399-404.

Evrard, S., Delanaye, P., Kamel, S., Cristol, J. P., Cavalier, E. and SFBC/SN (2015) 'Vascular calcification: from pathophysiology to biomarkers.' *Clinica Chimica Acta*, 438, January pp. 401-414.

Fakhry, M., Hamade, E., Badran, B., Buchet, R. and Magne, D. (2013) 'Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts.' *World Journal of Stem Cells*, 5(4) pp. 136-148.

Fakhry, M., Skafi, N., Fayyad-Kazan, M., Kobeissy, F., Hamade, E., Mebarek, S., Habib, A., Borghol, N., Zeidan, A., Magne, D., Fayyad-Kazan, H. and Badran, B. (2017) 'Characterization and assessment of potential microRNAs involved in phosphate-induced aortic calcification.' *Journal of Cellular Physiology*, 233(5), pp. 4056-4067.

Friedman, R. C., Farh, K. K., Burge, C. B. and Bartel, D. P. (2009) 'Most mammalian mRNAs are conserved targets of microRNAs.' *Genome Research*, 19(1) pp. 92-105.

Frismantiene, A., Philippova, M., Erne, P. and Resink, T. J. (2018) 'Smooth muscle cell-driven vascular diseases and molecular mechanisms of VSMC plasticity.' *Cellular Signalling*, 52, December, pp. 48-64.

Goettsch, C., Hutcheson, J. D. and Aikawa, E. (2013) 'MicroRNA in cardiovascular calcification: focus on targets and extracellular vesicle delivery mechanisms.' *Circulation Research*, 112(7) pp. 1073-1084.

Goettsch, C., Rauner, M., Pacyna, N., Hempel, U., Bornstein, S. R. and Hofbauer, L. C. (2011) 'miR-125b regulates calcification of vascular smooth muscle cells.' *The American Journal of Physiology*, 179(4) pp. 1594-1600.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A. and Enright, A. J. (2006) 'miRBase: microRNA sequences, targets and gene nomenclature.' *Nucleic Acids Research*, 34, January, pp. D140-144. [Online] [Accessed on 16th January 2018] DOI: 10.1093/nar/gkj112

Hartley, J. L., Temple, G. F. and Brasch, M. A. (2000) 'DNA cloning using in vitro site-specific recombination.' *Genome Research*, 10(11) pp. 1788-1795.

Hassan, M. Q., Gordon, J. A., Beloti, M. M., Croce, C. M., van Wijnen, A. J., Stein, J. L., Stein, G. S. and Lian, J. B. (2010) 'A network connecting Runx2, SATB2, and the miR-23a~27a~24-2 cluster regulates the osteoblast differentiation program.' *Proceedings of the National Academy of Sciences of the United States of America*, 107(46) pp. 19879-19884.

Hu, R., Li, H., Liu, W., Yang, L., Tan, Y. F. and Luo, X. H. (2010) 'Targeting miRNAs in osteoblast differentiation and bone formation.' *Expert Opinion of Therapeutic Targets*, 14(10) pp. 1109-1120.

Huang, J., Zhao, L., Xing, L. and Chen, D. (2010) 'MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation.' *Stem Cells*, 28(2) pp. 357-364.

Invitrogen. (2003) Gateway® Technology A universal technology to clone DNA sequences for functional analysis and expression in multiple systems. Carlsbad, USA: Invitrogen. [Online] [Accessed on 19th March 2018] URL: https://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf

Iyemere, V. P., Proudfoot, D., Weissberg, P. L. and Shanahan, C. M. (2006) 'Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification.' *Journal of Internal Medicine*, 260(3) pp. 192-210.

Jansen, F., Yang, X., Hoelscher, M., Cattelan, A., Schmitz, T., Proebsting, S., Wenzel, D., Vosen, S., Franklin, B. S., Fleischmann, B. K., Nickenig, G. and Werner, N. (2013) 'Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles.' *Circulation*, 128(18) pp. 2026-2038.

Kao, A. H., Wasko, M. C., Krishnaswami, S., Wagner, J., Edmundowicz, D., Shaw, P., Cunningham, A. L., Danchenko, N., Sutton-Tyrrell, K., Tracy, R. P., Kuller, L. H. and Manzi, S. (2008) 'C-reactive protein and coronary artery calcium in asymptomatic women with systemic lupus erythematosus or rheumatoid arthritis.' *American Journal of Cardiology*, 102(6) pp. 755-760.

Kapustin, A. N., Davies, J. D., Reynolds, J. L., McNair, R., Jones, G. T., Sidibe, A., Schurgers, L. J., Skepper, J. N., Proudfoot, D., Mayr, M. and Shanahan, C. M. (2011) 'Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization.' *Circulation Research*, 109(1) pp. e1-12. [Online] [Accessed on 30th June 2018] DOI: 10.1161/CIRCRESAHA.110.238808

Katano, H., Nishikawa, Y., Yamada, H., Yamada, K. and Mase, M. (2017) 'Differential Expression of microRNAs in Severely Calcified Carotid Plaques.' *Journal of Stroke and Cerebrovascular Diseases*, 27(1) pp. 108-117.

Kiani, A. N., Post, W. S., Magder, L. S. and Petri, M. (2011) 'Predictors of progression in atherosclerosis over 2 years in systemic lupus erythematosus.' *Rheumatology*, 50(11) pp. 2071-2079.

Kiani, A. N., Magder, L. S., Post, W. S., Szklo, M., Bathon, J. M., Schreiner, P. J., O'Leary, D. and Petri, M. (2015) 'Coronary calcification in SLE: comparison with the Multi-Ethnic Study of Atherosclerosis.' *Rheumatology*, 54(11) pp. 1976-1981.

Klages, N., Zufferey, R. and Trono, D. (2000) 'A stable system for the high-titer production of multiply attenuated lentiviral vectors.' *Molecular Therapy*, 2(2) pp. 170-176.

Krohn, J. B., Hutcheson, J. D., Martínez-Martínez, E. and Aikawa, E. (2016) 'Extracellular vesicles in cardiovascular calcification: expanding current paradigms.' *The Journal of Physiology*, 594(11) pp. 2895-2903.

Leopold, J. A. (2014) 'MicroRNAs Regulate Vascular Medial Calcification.' *Cells*, 3(4) pp. 963-980.

Leung, A. K. and Sharp, P. A. (2010) 'MicroRNA functions in stress responses.' *Molecular Cell*, 40(2) pp. 205-215.

Lian, J. B., Javed, A., Zaidi, S. K., Lengner, C., Montecino, M., van Wijnen, A. J., Stein, J. L. and Stein, G. S. (2004) 'Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors.' *Critical Reviews in Eukaryotic Gene Expression*, 14(1-2) pp. 1-41.

Liao, X. B., Zhang, Z. Y., Yuan, K., Liu, Y., Feng, X., Cui, R. R., Hu, Y. R., Yuan, Z. S., Gu, L., Li, S. J., Mao, D. A., Lu, Q., Zhou, X. M., de Jesus Perez, V. A. and Yuan, L. Q. (2013) 'MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells.' *Endocrinology*, 154(9) pp. 3344-3352.

Lin, M. E., Chen, T., Leaf, E. M., Speer, M. Y. and Giachelli, C. M. (2015) 'Runx2 Expression in Smooth Muscle Cells Is Required for Arterial Medial Calcification in Mice.' *The American Journal of Physiology*, 185(7) pp. 1958-1969.

Liu, X., Cheng, Y., Yang, J., Xu, L. and Zhang, C. (2012) 'Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application.' *Journal of Molecular and Cellular Cardiology*, 52(1) pp. 245-255.

Liu, Y., Wang, T., Yan, J., Jiagbogu, N., Heideman, D. A., Canfield, A. E. and Alexander, M. Y. (2011) 'HGF/c-Met signalling promotes Notch3 activation and human vascular smooth muscle cell osteogenic differentiation in vitro.' *Atherosclerosis*, 219(2) pp. 440-447.

Loebel, C., Czekanska, E. M., Bruderer, M., Salzmann, G., Alini, M. and Stoddart, M. J. (2015) 'In vitro osteogenic potential of human mesenchymal stem cells is predicted by Runx2/Sox9 ratio.' *Tissue Engineering Part A*, 21(1-2) pp. 115-123.

López-Pedrera, C., Aguirre, M., Barbarroja, N. and Cuadrado, M. J. (2010) 'Accelerated atherosclerosis in systemic lupus erythematosus: role of proinflammatory cytokines and therapeutic approaches.' *Journal of Biomedicine and Biotechnology*, 12(1) pp. 25-32.

Mackenzie, N. C., Staines, K. A., Zhu, D., Genever, P. and Macrae, V. E. (2014) 'miRNA-221 and miRNA-222 synergistically function to promote vascular calcification.' *Cell Biochemistry and Function*, 32(2) pp. 209-216.

Magder, L. S. and Petri, M. (2012) 'Incidence of and risk factors for adverse cardiovascular events among patients with systemic lupus erythematosus.' *American Journal of Epidemiology*, 176(8) pp. 708-719.

Mahmoud, A. M., Wilkinson, F. L., McCarthy, E. M., Moreno-Martinez, D., Langford-Smith, A., Romero, M., Duarte, J., Alexander, M. Y. (2017) 'Endothelial microparticles prevent lipid-induced endothelial damage via Akt/eNOS signaling and reduced oxidative stress.' *FASEB Journal*, 31(10) pp. 4636-4648.

Majeska, R. J. and Wuthier, R. E. (1975) 'Studies on matrix vesicles isolated from chick epiphyseal cartilage. Association of pyrophosphatase and ATPase activities with alkaline phosphatase.' *Biochima et Biophysia Acta*, 391(1) pp. 51-60.

Majesky, M. W. (2007) 'Developmental basis of vascular smooth muscle diversity.' *Arteriosclerosis, Thrombosis and Vascular Biology*, 27(6) pp. 1248-1258.

Manzi, S., Meilahn, E. N., Rairie, J. E., Conte, C. G., Medsger, T. A., Jansen-McWilliams, L., D'Agostino, R. B. and Kuller, L. H. (1997) 'Age-specific incidence rates of myocardial infarction and angina in women with systemic lupus erythematosus: comparison with the Framingham Study.' *American Journal of Epidemiology*, 145(5) pp. 408-415.

Mardis, E. and McCombie, W. R. (2017) 'Library Quantification: Fluorometric Quantitation of Double-Stranded or Single-Stranded DNA Samples Using the Qubit System.' *Cold Spring Harbour Protocols*, (6) June, p. pdb.prot094730. [Online] [Accessed on 14th March 201] DOI: 10.1101/pdb.prot094730

Mattick, J. S. and Makunin, I. V. (2006) 'Non-coding RNA.' *Human Molecular Genetics*, 15, April, pp. R17-29. [Online] [Accessed on 20th March 2018] DOI: 10.1093/hmg/ddl046

Mause, S. F. and Weber, C. (2010) 'Microparticles: protagonists of a novel communication network for intercellular information exchange.' *Circulation Research*, 107(9) pp. 1047-1057.

McCarthy, E. M., Wilkinson, F. L., Parker, B. and Alexander, M. Y. (2016) 'Endothelial microparticles: Pathogenic or passive players in endothelial dysfunction in autoimmune rheumatic diseases?' *Vascular Pharmacology*, 86, November, pp. 71-76.

McCarthy, E. M., Moreno-Martinez, D., Wilkinson, F. L., McHugh, N. J., Bruce, I. N., Pauling, J. D., Alexander, M. Y. and Parker, B. (2017) 'Microparticle subpopulations are potential markers of disease progression and vascular dysfunction across a spectrum of connective tissue disease.' *BBA Clinical*, 7, November, pp. 16-22.

McMahon, M., Hahn, B. H. and Skaggs, B. J. (2011) 'Systemic lupus erythematosus and cardiovascular disease: prediction and potential for therapeutic intervention.' *Expert Review of Clinical Immunology*, 7(2) pp. 227-241.

Nadra, I., Mason, J. C., Philippidis, P., Florey, O., Smythe, C. D., McCarthy, G. M., Landis, R. C. and Haskard, D. O. (2005) 'Proinflammatory activation of macrophages by basic calcium phosphate crystals via protein kinase C and MAP kinase pathways: a vicious cycle of inflammation and arterial calcification?' *Circulation Research*, 96(12) pp. 1248-1256.

Nanoudis, S., Pikilidou, M., Yavropoulou, M. and Zebekakis, P. (2017) 'The Role of MicroRNAs in Arterial Stiffness and Arterial Calcification. An Update and Review of the Literature.' *Frontiers in Genetics*, 8:209, pp. 1-11. [Online] [Accessed on 24th April 2018] DOI: 10.3389/fgene.2017.00209

New, S. E. and Aikawa, E. (2013) 'Role of extracellular vesicles in de novo mineralization: an additional novel mechanism of cardiovascular calcification.' *Arteriosclerosis, Thrombosis and Vascular Biology*, 33(8) pp. 1753-1758.

New, S. E., Goettsch, C., Aikawa, M., Marchini, J. F., Shibasaki, M., Yabusaki, K., Libby, P., Shanahan, C. M., Croce, K. and Aikawa, E. (2013) 'Macrophage-derived matrix vesicles: an alternative novel mechanism for microcalcification in atherosclerotic plaques.' *Circulation Research*, 113(1) pp. 72-77.

Nguyen, D. D. and Chang, S. (2017) 'Development of Novel Therapeutic Agents by Inhibition of Oncogenic MicroRNAs.' *International Journal of Molecular Sciences*, 19(1) pp. 1-17. [Online] [Accessed on 24th April 2018] DOI: 10.3390/ijms19010065

Owens, G. K., Kumar, M. S. and Wamhoff, B. R. (2004) 'Molecular regulation of vascular smooth muscle cell differentiation in development and disease.' *Physiological Reviews*, 84(3) pp. 767-801.

Pan, Q., Liu, H., Zheng, C., Zhao, Y., Liao, X., Wang, Y., Chen, Y., Zhao, B., Lazartigues, E., Yang, Y. and Ma, X. (2016) 'Microvesicles Derived from Inflammation-Challenged Endothelial Cells Modulate Vascular Smooth Muscle Cell Functions.' *Frontiers in Physiology*, 7:692, pp. 1-10. [Online] [Accessed on 20th April 2018] DOI: 10.3389/fphys.2016.00692

Parhami, F., Basseri, B., Hwang, J., Tintut, Y. and Demer, L. L. (2002) 'High-density lipoprotein regulates calcification of vascular cells.' *Circulation Research*, 91(7) pp. 570-576.

Parker, B., Al-Husain, A., Pemberton, P., Yates, A. P., Ho, P., Gorodkin, R., Teh, L. S., Alexander, M. Y. and Bruce, I. N. (2014) 'Suppression of inflammation reduces endothelial microparticles in active systemic lupus erythematosus.' *Annals of the Rheumatic Diseases*, 73(6) pp. 1144-1150.

Paudel, K. R., Panth, N. and Kim, D. W. (2016) 'Circulating Endothelial Microparticles: A Key Hallmark of Atherosclerosis Progression.' *Scientifica*, 2016, February, pp. 1-9. [Online] [Accessed on 21st April 2018] DOI: 10.1155/2016/8514056

Peeters, S. A., Engelen, L., Buijs, J., Chaturvedi, N., Fuller, J. H., Jorsal, A., Parving, H. H., Tarnow, L., Theilade, S., Rossing, P., Schalkwijk, C. G. and Stehouwer, C. D. A. (2017) 'Circulating matrix metalloproteinases are associated with arterial stiffness in patients with type 1 diabetes: pooled analysis of three cohort studies.' *Cardiovascular Diabetology*, 16(139) pp. 1-9. [Online] [Accessed on 3rd May 2018] DOI: 10.1186/s12933-017-0620-9

Preston, R. A., Jy, W., Jimenez, J. J., Mauro, L. M., Horstman, L. L., Valle, M., Aime, G. and Ahn, Y. S. (2003) 'Effects of severe hypertension on endothelial and platelet microparticles.' *Hypertension*, 41(2) pp. 211-217.

Reynolds, J. L., Joannides, A. J., Skepper, J. N., McNair, R., Schurgers, L. J., Proudfoot, D., Jahnen-Dechent, W., Weissberg, P. L. and Shanahan, C. M. (2004) 'Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD.' *Journals of the American Society of Nephrology*, 15(11) pp. 2857-2867.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Zhang, M., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L. and Van Parijs, L. (2003) 'A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference.' *Nature Genetics*, 33(3) pp. 401-406.

Sabio, J. M., Vargas-Hitos, J. A., Navarrete-Navarrete, N., Mediavilla, J. D., Jiménez-Jáimez, J., Díaz-Chamorro, A., Jiménez-Alonso, J. and Nieves, G. L. V. d. l. (2011) 'Prevalence of and factors associated with hypertension in young and old women with systemic lupus erythematosus.' *The Journal of Rheumatology*, 38(6) pp. 1026-1032.

Schiro, A., Wilkinson, F. L., Weston, R., Smyth, J. V., Serracino-Inglott, F. and Alexander, M. Y. (2014) 'Endothelial microparticles as conveyors of information in atherosclerotic disease.' *Atherosclerosis*, 234(2) pp. 295-302.

Segura, M. M., Garnier, A., Durocher, Y., Coelho, H. and Kamen, A. (2007) 'Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification.' *Biotechnology and Bioengineering*, 98(4) pp. 789-799.

Shanahan, C. M., Cary, N. R., Salisbury, J. R., Proudfoot, D., Weissberg, P. L. and Edmonds, M. E. (1999) 'Medial localization of mineralization-regulating proteins in association with Mönckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification.' *Circulation*, 100(21) pp. 2168-2176.

Shoenfeld, Y., Gerli, R., Doria, A., Matsuura, E., Cerinic, M. M., Ronda, N., Jara, L. J., Abu-Shakra, M., Meroni, P. L. and Sherer, Y. (2005) 'Accelerated atherosclerosis in autoimmune rheumatic diseases.' *Circulation*, 112(21) pp. 3337-3347.

Sinicato, N. A., da Silva Cardoso, P. A. and Appenzeller, S. (2013) 'Risk factors in cardiovascular disease in systemic lupus erythematosus.' *Current Cariology Reviews*, 9(1) pp. 15-19.

Sinning, J. M., Losch, J., Walenta, K., Böhm, M., Nickenig, G. and Werner, N. (2011) 'Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes.' *European Heart Journal*, 32(16) pp. 2034-2041.

Speer, M. Y., Li, X., Hiremath, P. G. and Giachelli, C. M. (2010) 'Runx2/Cbfa1, but not loss of myocardin, is required for smooth muscle cell lineage reprogramming toward osteochondrogenesis.' *Journal of Cellular Biochemistry*, 110(4) pp. 935-947.

Steinberg, D. (2009) 'The LDL modification hypothesis of atherogenesis: an update.' *The Journal of Lipid Research*, 50, April, pp. S376-381. [Online] [Accessed on 2nd February 2018] DOI: 10.1194/jlr.R800087-JLR200

Steitz, S. A., Speer, M. Y., Curinga, G., Yang, H. Y., Haynes, P., Aebersold, R., Schinke, T., Karsenty, G. and Giachelli, C. M. (2001) 'Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers.' *Circulation Research*, 89(12) pp. 1147-1154.

Sun, Y., Byon, C. H., Yuan, K., Chen, J., Mao, X., Heath, J. M., Javed, A., Zhang, K., Anderson, P. G. and Chen, Y. (2012) 'Smooth muscle cell-specific runx2 deficiency inhibits vascular calcification.' *Circulation Research*, 111(5) pp. 543-552.

Takemura, A., Iijima, K., Ota, H., Son, B. K., Ito, Y., Ogawa, S., Eto, M., Akishita, M. and Ouchi, Y. (2011) 'Sirtuin 1 retards hyperphosphatemia-induced calcification of vascular smooth muscle cells.' *Arteriosclerosis, Thrombosis and Vascular Biology*, 31(9) pp. 2054-2062.

Tintut, Y., Patel, J., Parhami, F. and Demer, L. L. (2000) 'Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway.' *Circulation*, 102(21) pp. 2636-2642.

Tiscornia, G., Singer, O. and Verma, I. M. (2006) 'Production and purification of lentiviral vectors.' *Nature Protocols*, 1(1) pp. 241-245.

Tyson, K. L., Reynolds, J. L., McNair, R., Zhang, Q., Weissberg, P. L. and Shanahan, C. M. (2003) 'Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification.' *Arteriosclerosis, Thrombosis and Vascular Biology*, 23(3) pp. 489-494.

Urowitz, M. B., Gladman, D., Ibañez, D., Fortin, P., Sanchez-Guerrero, J., Bae, S., Clarke, A., Bernatsky, S., Gordon, C., Hanly, J., Wallace, D., Isenberg, D., Ginzler, E., Merrill, J., Alarcón, G. S., Steinsson, K., Petri, M., Dooley, M. A., Bruce, I., Manzi, S., Khamashta, M., Ramsey-Goldman, R., Zoma, A., Sturfelt, G., Nived, O., Maddison, P., Font, J., van Vollenhoven, R., Aranow, C., Kalunian, K., Stoll, T. and Clinics, S. L. I. C. (2008) 'Accumulation of coronary artery disease risk factors over three years: data from an international inception cohort.' *Arthritis & Rheumatology*, 59(2) pp. 176-180.

Vlachos, I. S., Paraskevopoulou, M. D., Karagkouni, D., Georgakilas, G., Vergoulis, T., Kanellos, I., Anastasopoulos, I. L., Maniou, S., Karathanou, K., Kalfakakou, D., Fevgas, A., Dalamagas, T. and Hatzigeorgiou, A. G. (2015) 'DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions.' *Nucleic Acids Research*, 43, January, pp. D153-159. [Online] [Accessed on 23rd January 2018] DOI: 10.1093/nar/gku1215.

Wahid, F., Shehzad, A., Khan, T. and Kim, Y. Y. (2010) 'MicroRNAs: synthesis, mechanism, function, and recent clinical trials.' *Biochima et Biophysia Acta*, 1803(11) pp. 1231-1243.

Wang, Y., Chen, S., Deng, C., Li, F., Hu, X., Shi, F. and Dong, N. (2015) 'MicroRNA-204 Targets Runx2 to Attenuate BMP-2-induced Osteoblast Differentiation of Human Aortic Valve Interstitial Cells.' *Journal of Cardiovascular Pharmacology*, 66(1) pp. 63-71.

Watson, K. E., Boström, K., Ravindranath, R., Lam, T., Norton, B. and Demer, L. L. (1994) 'TGF-beta 1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify.' *Journal of Clinical Investigation*, 93(5) pp. 2106-2113.

Wojciechowska, A., Braniewska, A. and Kozar-Kamińska, K. (2017) 'MicroRNA in cardiovascular biology and disease.' *Advances in Clinical and Experimental Medicine*, 26(5) pp. 865-874.

Worth, N. F., Rolfe, B. E., Song, J. and Campbell, G. R. (2001) 'Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganisation of contractile and cytoskeletal proteins.' *Cell Motlity and the Cytoskeleton*, 49(3) pp. 130-145.

Wu, G. C., Liu, H. R., Leng, R. X., Li, X. P., Li, X. M., Pan, H. F. and Ye, D. Q. (2016) 'Subclinical atherosclerosis in patients with systemic lupus erythematosus: A systemic review and meta-analysis.' *Autoimmunity Reviews*, 15(1) pp. 22-37.

Yan, J, Stringer, S. E., Hamilton, A., Charlton-Menys, V., Götting, C., Müller, B., Aeschlimann, D., Alexander, M. Y. (2011) 'Decorin GAG synthesis and TGF-β signaling mediate Ox-LDL-induced mineralization of human vascular smooth muscle cells. ' *Arteriosclerosis, Thrombosis and Vascular Biology*, 31(3) pp. 608-615.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. L. (2012) 'Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction.' *BMC Bioinformatics*, 13(134) p. 1-11. [Online] [Accessed on 1st February 2018] DOI: 10.1186/1471-2105-13-134

Zamurovic, N., Cappellen, D., Rohner, D. and Susa, M. (2004) 'Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-

induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity.' *The Journal of Biological Chemistry*, 279(36) pp. 37704-37715.

Zhang, Y., Xie, R. L., Croce, C. M., Stein, J. L., Lian, J. B., van Wijnen, A. J. and Stein, G. S. (2011) 'A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2.' *Proceedings of the National Academy of Sciences of the United States of America*, 108(24) pp. 9863-9868.

Zhang, Y., Xie, R. L., Gordon, J., LeBlanc, K., Stein, J. L., Lian, J. B., van Wijnen, A. J. and Stein, G. S. (2012) 'Control of mesenchymal lineage progression by microRNAs targeting skeletal gene regulators Trps1 and Runx2.' *The Journal of Biological Chemistry*, 287(26) pp. 21926-21935.

Zhang, Y., Yang, X., Bian, F., Wu, P., Xing, S., Xu, G., Li, W., Chi, J., Ouyang, C., Zheng, T., Wu, D., Li, Y. and Jin, S. (2014) 'TNF-α promotes early atherosclerosis by increasing transcytosis of LDL across endothelial cells: crosstalk between NF-κB and PPAR-γ.' *Journal of Molecular and Cellular Cardiology*, 72, March, pp. 85-94.

Zhao, Y. G., Meng, F. X., Li, B. W., Sheng, Y. M., Liu, M. M., Wang, B., Li, H. W. and Xiu, R. J. (2016) 'Gelatinases promote calcification of vascular smooth muscle cells by up-regulating bone morphogenetic protein-2.' *Biochemical and Biophysical Research Communications*, 470(2) pp. 287-293.

Zhou, G., Zheng, Q., Engin, F., Munivez, E., Chen, Y., Sebald, E., Krakow, D. and Lee, B. (2006) 'Dominance of SOX9 function over RUNX2 during skeletogenesis.' *Proceedings of the National Academy of Sciences of the United States of America*, 103(50) pp. 19004-19009.